UNIVERSITY OF ALBERTA

STRUCTURE, DISTRIBUTION AND PHYSIOLOGICAL FUNCTIONS OF GHRELIN AND GALANIN IN GOLDFISH

by

SURAJLAL UNNIAPPAN MOOTHATHU ©

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ABSTRACT

Ghrelin and galanin are two endocrine factors involved in the regulation of many physiological functions in mammals. However, in non-mammalian vertebrates both ghrelin and galanin are not well studied. The objectives of the projects undertaken during my Ph.D. program were to study the structure, distribution and physiological functions of ghrelin and galanin in goldfish, *Carassius auratus*.

The gene encoding ghrelin in goldfish has been identified using molecular biology tools. Using bioinformatics tools, the peptide structure was deduced from the nucleotide sequence. Two putative ghrelin peptides, a nineteen amino acid, long ghrelin ($gGRL_{[19]}$) and a twelve amino acid, short ghrelin ($gGRL_{[12]}$) are present in goldfish. Ghrelin mRNA expression has been found in the gut, spleen, liver and brain of goldfish. Both intracerebroventricular (ICV) and intraperitoneal (IP) injections of human ghrelin, $gGRL_{[19]}$ and $gGRL_{[12]}$ stimulates food intake in goldfish. A post-meal decrease and a starvation-induced increase in ghrelin mRNA expression and serum ghrelin levels were found in goldfish. These results indicate that ghrelin is a novel appetite stimulatory factor (orexigen) in goldfish. Ghrelin stimulates both growth hormone (GH) and luteinizing hormone (LH) release from goldfish pituitary cells *in vitro*. ICV and IP administration of ghrelin stimulates both GH and LH release *in vivo* in goldfish. These results, described in section A of this thesis, indicate that ghrelin is a multifunctional hormone in goldfish.

Two genes encoding galanin are present in the goldfish. Three alternative splice variant mRNA forms from galanin gene 1 and two splice variant forms from galanin gene 2 were discovered. The amino acid sequences predicted using bioinformatics tools indicate that five preprogalanin peptides are present in the goldfish. Using *in situ* hybridization, galanin mRNA expression has been identified in brain nuclei involved in the regulation of food intake and pituitary hormone secretion in goldfish. A post-meal decrease in galanin mRNA expression was found in the goldfish. The results, discussed in section B of this thesis, indicate that galanin is involved in the regulation of multiple physiological functions in goldfish.

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LIST OF ABBREVIATIONS

Abbreviation	Description
AC	adenylate cyclase
AGRP	agouti related peptide
AVP	arginine vasopressin
BBS	bombesin
C	degree celsius
CART	cocaine and amphetamine regulated transcript
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary
CRF	corticotropin releasing factor
DNA	deoxyribonucleic acid
GABA	gamma amino butyric acid
GAL-R	galanin receptor
GGDT transcript	ghrelin gene derived
gGRL	goldfish ghrelin
GH	growth hormone
GHS	growth hormone secretagogoue receptor
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing peptide
GLP	glucagon-like peptide
GnRH peptide	gonadotropin releasing
GPCR	G-protein coupled receptor
HEK	human embryonic kidney
hGRL	human ghrelin
HPLC	high performance liquid chormatography
ICV	intracerebroventricular
IP	intraperitoneal
IRS	insulin receptor signaling
IV	intravenous
LH	luteinizing hormone

MCH	melanin concentrating hormone
MC-4-R	melanocoritn - 4 - receptor
mRNA	messenger ribonucleic acid
NAT	nucleus anterior tuberis
NPP	nucleus prepoticus paraventicularis
NPY	neuropeptide Y
NPO	nucleus prepoticus
NLTl	nucelus lateral tuberis
NRL	nucleus recessus lateralis
PACAP	pituitary adenylate cyclase activating polypeptide
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PKC	protein kinase C
PKA	protein kinase A
PLC	phospholipase C
POMC	proopiomelanocortin
ppCCK	preprocholecystokinin
PRL	prolactin
PVN	paraventricular nucleus
RACE	rapid amplification of cDNA ends
RIA	radioimmunoassay
RNase	ribonuclease
RT	reverse transcription
RQ	respiratory quotient
SS	somatostatin
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
UTR	untranslated region
VSCC	voltage sensitive calcium channels
Vv	ventral telencephalon

CHAPTER 1

INTRODUCTION – LITERATURE REVIEW

SECTION A - GHRELIN

1. History of Ghrelin

1.1. Growth hormone secretagogues

Growth hormone secretagogues (GHS) are the group of synthetic peptidyl and non-peptidyl compounds that are capable of stimulating growth hormone (GH) secretion. Dr. Cyril Y.Bowers and colleagues at Tulane Medical School discovered the first GHS, growth hormone releasing peptide (GHRP) in 1976. Dr. Bower's group derived these small molecules from metenkephalin, using low-energy conformation calculations, computer modeling and structural modifications (Bowers et al., 1980). During the early eighties the first highly potent GHS named GHRP-6 was developed (Bowers et al., 1981). The actions of GHRP-6 as a potent stimulator of GH have been demonstrated. However, the discovery of growth hormone releasing hormone (GHRH) in 1982 as the first endogenous GH releasing factor shifted the interest away from GHRP-6. From then onwards, the idea of GH secretion from somatotropes being controlled by two hormones: GHRH, which is stimulatory and somatostatin (SS), which is inhibitory became prevalent. Purification and characterization of both peptides from various species helped the growth of this concept. However, Dr.Bowers and his research group at the Tulane Medical School as well as several other researchers around the world continued the search for more desirable synthetic GHS, the endogenous GHS and its receptor.

1.1.1. Physiological functions of GHS

Several studies were conducted to identify the structural, biochemical and physical properties of GHS. A vast amount of literature is also available on the biological actions of GHS and its potential therapeutic use. However, a detailed literature review on those aspects of GHS is not the aim of this chapter. I will only briefly discuss the role of GHS on GH release and food intake, two of the major physiological functions of GHS.

1.1.1.a. GH release

Recent studies by Tannenbaum and Bowers (2001) demonstrated the role of GHRP-6 on GH secretion and its interaction with the GHRH and SS. Intravenous administration of GHRP-6 stimulates GH secretion in rats. Immunoneutralization of GHRH blunted GHRP-6 induced GH release. This result suggests that the GHS induced GH secretion may be mediated by GHRH. GHS-receptor (GHS-R) mRNA expression in the GHRH neurons of the arcuate nuclei and the ventromedial nucleus of rats has been demonstrated. GHS may directly act on the GHS-R in the GHRH neurons to stimulate GHRH secretion resulting in a subsequent increase in GH secretion (Tannenbaum and Bowers, 2001).

1.1.2.b. Food intake

The GHS KP-102 stimulates food intake in fasted and fed rats (Okada et al., 1996, Kuriyama et al., 2000). Intracerebroventricular (ICV) administration of KP-102 increased food intake in freely fed rats, but not in fasted rats. Continuous infusion of KP-102 for 6 days stimulated food intake in stressed and non-stressed

rats (Kuriyama et al., 2000). They hypothesized that neuropeptide Y (NPY) neurons in the arcuate nuclei mediate the stimulation of feeding behavior by GHS, although, there is no additional evidence to support this hypothesis.

2. GHS receptor – A novel, orphan receptor

Dr. Andrew Howard and colleagues at the Merck research laboratories were successful in cloning a G protein coupled receptor from human and swine hypothalamus and pituitary using the expression cloning strategy (Howard et al., 1996). The GHS MK-0677 bound to this receptor expressed in *Xenopus* oocytes, signaled through the phospholipase C pathway and induced Ca^{2+}-activated chloride currents. Since GHS can bind to this receptor and cause biological effects it was named as the growth hormone secretagogue receptor (GHS-R). However, the identity of the endogenous ligand for the GHS-R remained unknown.

Two types of GHS-R were identified from the swine pituitary complementary deoxyribonucleic acid (cDNA) library, a long form GHS-R called the GHS-R1a and a short form, called the GHS-R1b (Smith et al., 1996). GHS-R1a belongs to the G-protein coupled receptor (GPCR) family and it has seven transmembrane domains and the TRY signature motif. The GHS-R1b is also encoded in the GHS-R1a gene. However, it lacks the transmembrane domains 6 and 7. GHS-R1b expressed in HEK 293 and COS7 cell lines was devoid of functional activity (Howard et al., 1996). GHS-R1b may be a functionally inactive splice variant. Chromosomal localization of human GHS-R is in 3Q26.2 (Smith et al., 1999). Studies in rhesus monkey and in rats using electrophysiology, *in situ* hybridization and *c-fos* expression have shown

that GHS-R is located in the arcuate nuclei suggesting the possible action of GHS on arcuate nuclei GHRH neurons and NPY/agouti related protein (AGRP) neurons. GHS-R mRNA expression has been found in the anterior pituitary gland, suprachiasmatic nucleus, supraoptic nucleus, ventromedial hypothalamus, dentate gyrus, tuberomedullary nucleus, pars compacta of substantia nigra, the ventral tegmental area, dorsal raphe nuclei, and median raphe nuclei (Smith et al., 1999). Gnanapavan et al. (2002) detected the expression of GHS-R mRNA in the pituitary, thyroid gland, pancreas, spleen, myocardium and adrenal gland.

Adenosine can bind to the GHS-R1a, which suggests that it is an agonist of the GHS-R1a (Tullin et al., 2000, Smith et al., 2000). However, adenosine did not stimulate GH release from the pituitary (Tullin et al., 2000, Smith et al., 2000). Smith et al., (2000) found that adenosine binds to a binding site that is different from where the synthetic GHSs, GHRP-6 and MK-0677 bind.

3. GHS-R and actions of GHS in non-mammalian vertebrates

3.1. Avians

Recently, two independent studies reported the structure and distribution of GHS-R in chicken. Geelissen et al., (2003) identified two partial GHS-R cDNAs in chicken. The reported GHS-R1a and GHS-R1c, its alternative splice variant lacking 16 amino acids from the transmembrane domain 6 (TM6), have respectively, 81 and 78% homology with the human GHS-R. Tanaka et al. (2003) independently discovered two similar GHS-R cDNAs in the chicken. Both studies found high expression of GHS-R mRNAs in the brain and pituitary. GHS-R mRNA is also

present widely in other tissues of chicken. Unlike in mammals, ICV injections of a synthetic GHS, KP-102, inhibited food intake in neonatal chicks (Saito et al., 2002).

3.2. Fish

GHS-R has been identified in the pufferfish, *Spheroides nephelus* (Palyha et al., 2000). Pufferfish GHS-R has 58% identity with the human GHS-R. GHS activated the pufferfish GHS-R expressed in HEK cells. These results suggest that the structure of GHS-R is highly conserved in fish compared to mammals. Shepherd et al. (2000) studied the role of synthetic GHS KP-102 on GH secretion in tilapia, *Oreochromis mossambicus.* IP injections of KP-102 at concentrations of 1 ng/g and 10 ng/g of body weight increased plasma GH levels. No changes in plasma prolactin levels were found after the GHS treatment. The identification of GHS-R from the pufferfish and an *in vivo* effect for the synthetic ligand in inducing GH release in a teleost species suggest the presence of an endogenous ligand for the GHS-R in fish.

4. The discovery of ghrelin

By 1999, the GHS and GHS-R receptor had become a broad area of research with immense interest in studying the potential functions and clinical use of the GHS. Several researchers were using a combination of biochemistry, peptide biology, cell biology and physiology to identify the endogenous ligand of GHS-R. Using the orphan receptor, GHS-R and the reverse pharmacology approach, Kojima et al. (1999), successfully purified, characterized and cloned the cDNA encoding a

novel protein, "ghrelin" (from the proto-indo-european word "ghre"which means "to grow"), the first endogenous ligand for GHS-R.

4.1. Structure of ghrelin

4.1.1. Gene

Structure of ghrelin gene was first reported from humans (Wajnaranjch et al., 2000). Human ghrelin gene comprises of 3 introns and 4 exons. Human ghrelin gene is located in the chromosome 3p25-26. Recently, Tanaka et al. (2001a) reported the structure of mouse ghrelin gene. They found the presence of a short non-coding first exon in the mouse gene, the organization being comprised of 5 exons and 4 introns. The first exon is a 19 bp, non coding region of the cDNA. A sequence comparison found that the human and rat ghrelin genes have a similar, highly homologous 19 bp region and the TATA box like sequence, TATATAA, in the 5' promoter region. This suggests that the intron:exon organization and the promoter region are conserved in the mammalian ghrelin genes.

4.1.2. Peptide

Ghrelin is the first identified endogenous ligand of GHS-R (Kojima, 1999). It is a 28 amino acid peptide in rats and humans. It has a unique structure with a lipid *n-octanoyl* ($O{=}CO\text{-}[CH_2]_6\text{-}CH_3$) modification at the third serine residue. The n-*octanoyl* modification is essential for the stimulation of GH secretion. Tomasetto et al. (1999) discovered a motilin-related peptide, which has a 100% sequence similarity with ghrelin. However, later it was confirmed that both motilin related peptide (MTLRP) and ghrelin are the same (Folwacnzy et al., 2001). The octanoyl

group is essential for the biological activity of the peptide and ghrelin without the acyl group (des-acyl ghrelin) is biologically inactive. Des-acyl ghrelin has been identified from rats and humans. Hosoda et al. (2003) found multiple, biologically active, ghrelin derived molecules produced by posttranslational modifications in humans. They are octanoyl ghrelin (1-27), decanoyl (O-CO-C_9H_{19}) ghrelin and decenoyl (modified with the decenoic acid form of O-CO-C_9H_{19}) ghrelin.

Bednarek et al. (2000) identified the minimum sequence of ghrelin required to activate the GHS-R1a. They found that the first four amino acids "GSS(*n-octanoyl*)F" can stimulate intracellular Ca^{2+} concentrations in HEK-293 cells expressing human GHS-R1a. These results suggest that the full-length ghrelin is not necessary for the activation of GHS-R1a. The first four amino acids in the n-terminal with the octanoyl modification was named as the "essential core" or the "active core" of the ghrelin peptide. However, studies by Torsello et al. (2002) found that short ghrelin peptides comprising of the first 4-5 amino acids of ghrelin cannot displace full-length ghrelin binding to GHS-R1a. They also found that the subcutaneous administration of the short peptides does not stimulate GH release in neonatal rats. These results suggest that the effective binding of the small peptides stimulating the GHS-R1a *in vitro* does not reflect its ability to induce biological actions *in vivo*.

Matsumoto et al. (2001a) added more evidence to the "active core" concept of the ghrelin peptide. They demonstrated that the first glycine and the second serine act as a spacer, providing necessary space between the octanoyl group and the n-amino group. Biochemical analysis found that the two amino acids in the n-terminal

contribute to the "biologically active geometry" of the peptide by properly making the conformation of amino residues in positions 3 and 4. Matsumoto et al. (2001b) also found that modifications other than the n-octanoylation (eg: decanoyl, lauroyl [O-CO-$C_{11}H_{23}$], palmitoyl [O-CO-$C_{15}H_{31}$], 4-methylpentanoyl [O-CO-$CH_2CH_2CH(CH_3)_2$]) in the third serine can result in biological activity. Mastumoto et al. (2001b) demonstrated that substitution of the aliphatic moiety in the third serine with an aromatic group can result in a biologically active ghrelin, suggesting that the hydrophobicity of the side chain on the third serine moiety is essential for biological activity, irrespective of its aliphatic or aromatic nature. Palucki et al. (2001) tested the biological efficiency of ghrelin mimetic peptides that are spiro(indoline-3',4'-piperidine) analogues of ghrelin and found that these analogues have a similar potency to ghrelin. The ghrelin mimetics increased intracellular calcium levels in HEK-293 cells expressing GHS-R1a. Results of the circular dichorism (CD) and nuclear magnetic resonance (NMR) spectroscopy studies suggest that human ghrelin and its truncated analogues do not possess well defined secondary or tertiary structures in solution (Elipe et al., 2001).

Banks et al. (2002) provided the first evidence for the bidirectional transport of ghrelin across the blood-brain barrier (BBB) in mice. From their results, it is clear that n-octanoylated human ghrelin and mouse ghrelin can cross the BBB in both directions. The extent and direction of ghrelin transport depends on the presence of the acyl modification and its amino acid sequence.

4.1.3. [Des-Glu]^{14} ghrelin

Hosoda et al. (2000a) purified and characterized the second endogenous ligand for rat GHS-R, [Des-Glu]^{14} ghrelin (GSSFLSPEHQKAQRKESKKPPAKLQPR). This 27 amino acid peptide has a sequence similar to ghrelin except for the absence of one amino acid, glutamine at position 14. They also demonstrated that [Des-Glu]^{14} ghrelin is a product of the alternate splicing of rat ghrelin mRNA. Hosoda et al. (2000a) studied the actions of [Des-Glu]^{14} ghrelin *in vivo* in rat. Their results show that intravenous injection of [Des-Glu]^{14} ghrelin increases plasma GH concentrations with a maximum effect produced at 10-20 minutes postinjection.

4.1.4. Ghrelin gene derived transcript (GGDT)

A testis specific ghrelin gene derived transcript (GGDT) has been identified from the mouse (Tanaka et al., 2001b). 252 bp of the GGDT cDNA are identical to the sequence encoded by the exons 4 and 5 of mouse ghrelin gene. However, the 54 amino acids encoded by the GGDT do not contain a signal peptide region. Functional significance of this peptide is unknown.

5. Distribution of ghrelin

5.1. mRNA

Using Northern blot analysis, ghrelin expression was detected in the stomach of rats (Kojima et al., 1999). In mouse, a wide distribution of ghrelin mRNA in tissues including brain, stomach and kidneys has been detected using RT-PCR (Hosoda et al., 2000b). Gualillo et al. (2000) showed the presence of ghrelin gene in

human placenta by RT-PCR, suggesting a role for ghrelin in fetal growth and development. Ghrelin mRNA has been detected in the osteoblastic cells, and kidney glomerulus and glomerular cells (Mori et al., 2000). Ghrelin mRNA has been identified in stomach, small and large intestines, pancreas, liver, heart, lung, white adipose tissue, hypothalamus, pituitary and the forebrain of rats (Date et al., 2000).

Most recent RT-PCR analysis by Gnanapavan et al. (2002) reported extensive distribution of ghrelin mRNA in humans. They found ghrelin mRNA expression in the stomach, adrenal gland, atrium, breast, buccal mucosa, esophagus, fallopian tube, fat tissue, gall bladder, lymphocytes, ileum, kidney, left colon, liver, lungs, lymph node, muscle, myocardium, ovary, pancreas, pituitary, placenta, prostate, right colon, skin, spleen, testis, thyroid and vein. Ghrelin mRNA expression has been detected in human T cells, B cells and neutrophils obtained from both cell lines and healthy human volunteers, suggesting a role for ghrelin in immune functions (Hattori et al., 2001). Although the relevance of the extensive expression of ghrelin mRNA is unknown, all these results suggest a broad physiological role for ghrelin in humans. Interestingly, there are no reports on the Northern blot detection of ghrelin mRNA in tissues other than the stomach, suggesting that stomach is the major site of ghrelin production.

Using *in situ* hybridization, Kamegi et al. (2000) studied the effects of ICV administration of ghrelin on SS, GHRH and NPY mRNA expression in the rat brain. There is a significant increase in NPY mRNA expression in the arcuate nuclei. Their results suggest that the central administration of ghrelin does not have any effect on

SS or GHRH mRNA expression in paraventricular and arcuate nuclei. These results suggest that the physiological role of ghrelin in the brain may be mediated by NPY. Recent colocalization of GHS-R with NPY mRNA in the arcuate nuclei of rat (Willesen et al., 1999) and induction of *c-fos* mRNA expression in arcuate nuclei by GHS (Sirinathsinghji et al., 1995) further substantiates the possible involvement of NPY in mediating the actions of ghrelin on food intake.

5.2. Peptide

Immunohistochemical localization of ghrelin has been investigated in an array of tissues in various mammals. Kojima et al. (1999) first reported the presence of ghrelin in the arcuate nuclei of rat brain. Recently, Lu et al. (2002) identified extensive ghrelin immunoreactivity in the ventral part of the arcuate nuclei. A more recent study found ghrelin immunoreactivity in the rat brain (Cowley et al., 2003). In the rat hypothalamus, ghrelin immunoreactive perikarya were found in the internuclear spaces between the lateral hypothalamus, arcuate, ventromedial, dorsomedial and paraventrcular nuclei. Ghrelin immunoreactive neurons were also found on the ependymal layer of the third ventricle (Cowley et al., 2003). Ghrelin neurons are in contact with the cell bodies and dendrites on NPY/AGRP and proopiomelanocortin (POMC) neurons in the hypothalamus. Ghrelin neurons also have contact with gamma amino butyric acid (GABA) axon terminals in the arcuate nuclei and the periventricular hypothalamus, and also with the corticotropin releasing factor (CRF) neurons in the periventricular hypothalamus (Cowley et al., 2003).

Date et al. (2000) identified the ghrelin producing cells in the stomach of rats and humans. Ghrelin is present in the stomach and extend to the colon with the highest immunoreactivity in the gastric fundus. About 20% of the endocrine cells in the oxyntic gland of rats and humans stained positive for ghrelin. They were electron-dense granule filled, round, compact cells, called the X/A cells with unknown hormonal secretions. The ghrelin positive X/A cells were renamed ghrelin cells. Neither human nor rat ghrelin cells colocalize SS and serotonin. Recently, Sakata et al. (2002a) described the morphological characters of Gr cells in the gastrointestinal tract of rat. They classified the ghrelin cells as two groups: (1) the open-type cells, found abundantly in the duodenum, jejunum, ileum, cecum and colon, which are elongated or triangular with their apical cytoplasmic extensions contacting the lumen of the intestine and (2) the closed-type cells, which are small and round in nature, found in the stomach. Sakata et al. (2002b) detected differences in the postnatal expression of ghrelin cells in the female and male rats. There is an increase in age-dependent ghrelin cell density in the fundic gland of male rats compared to the female rats, in which there is no change in ghrelin cell density. Ghrelin cell density in female rats was higher compared to the male rats until the 3 week postnatal period.

Gaytan et al. (2003) localized ghrelin immunoreactivity in the human ovary. Ghrelin is present in the ovarian hilus interstitial cells while ghrelin immunoreactivity was not detected in the ovarian follicles at any time during development. Ghrelin-ir was present in the corpus luteum. These results suggest a

potential role for ghrelin in the ovarian physiology and reproduction. Ghrelin-immunoreactivity has been detected in the islet cells of human pancreas (Wierup et al., 2002), islet cell tumors (Iwakura et al., 2002), human cord blood (Chanoine et al., 2002), placenta (Gualillo et al., 2000), pituitary and neuroendocrine tumors (Korbonits et al., 2001), breast cell carcinoma and cell lines (Cassoni et al., 2001), endocrine tumors of the stomach and intestine (Papotti et al., 2001), human thyroid cell carcinoma cell line (Kanamoto et al., 2001), and fetal, infant and adult human lung (Volante et al., 2002). These results provide evidence for ghrelin's potential role in the physiology and pathophysiology of a wide range of endocrine and non-endocrine tissues in mammals.

6. Biological actions of ghrelin

Ghrelin is involved in the regulation of a number of physiological functions. The aim of this section is to give an overview of ghrelin's role in the regulation of GH release and food intake. Detailed discussions of relevant aspects of the biological actions of ghrelin will be included in the following chapters.

6.1. In mammals

6.1.1. GH secretion

The role of ghrelin in the regulation of GH secretion has been studied in rats, pigs, cows and humans. In rats, ghrelin stimulates GH secretion *in vitro* in a dose dependent manner (Kojima et al., 1999). Ghrelin stimulates GH release from the perifused anterior pituitary cells of rats (Yamazaki et al., 2002). Ghrelin stimulates GH release from porcine (Glavaski-Joksimovic et al., 2003) and bovine (Hashizume

et al., 2003) somatotropes in static culture. These results indicate that ghrelin can act directly on the pituitary to regulate GH release in mammals.

Ghrelin stimulates GH release *in vivo*. IP injections of ghrelin stimulate GH release in rats in a dose dependent manner (Kojima et al., 1999, Dieguez et al., 2000). Wren et al. (2000) found an increase in plasma GH levels after the ICV or IP administration of ghrelin in rats. Continuous ICV infusion of ghrelin for 12 days increased the plasma GH concentration on day 6 (Nakazato et al., 2000). However, this effect was not observed on day 12. SS inhibits the ghrelin induced GH secretion (Sakai et al., 2002) *in vitro*. No additive response was found when cells were perifused with GHRH and ghrelin. The potency of the GH-releasing effect of ghrelin is comparable to that of GHRH. Nakao et al. (2001) demonstrated that intravenous administration of a low dose of ghrelin can increase GH release in humans. Cortistatin - 14, a novel, natural SRIF analogue, decreases the ghrelin-induced GH secretion (Nakao et al., 2001). In humans, intravenous administration of SS-14 and cortistatin decreases ghrelin secretion (Ghigo et al., 2002). Infusion of GHRH increases the expression of ghrelin mRNA in the pituitary of rats (Kamegai et al., 2001a). This event may lead to an increase in ghrelin levels that can result in an increase in GH secretion from the somatotropes due to ghrelin's autocrine or paracrine action. These results indicate that ghrelin stimulates GH release *in vitro* and *in vivo* in mammals. Furthermore, the data imply that somatostatin is a potent inhibitor of ghrelin's effects on GH release in mammals.

In porcine somatotropes, ghrelin-stimulated GH release was abolished by the blockade of either phospholipase C (PLC) or protein kinase C (PKC), suggesting a role for this pathway in mediating ghrelin's effects on GH release (Malagon et al., 2003). Inhibition of either adenylate cyclase (AC) or protein kinase A (PKA) also inhibited ghrelin's effects on GH release. Furthermore, the blockade of the entry of extracellular calcium through the L-type voltage sensitive calcium channels (VSCC) inhibited ghrelin's effects on GH release from porcine somatotropes. Thus, it is possible to conclude that at least in porcine somatotropes, a coordinated action of the three intracellular signaling pathways (AC/PKA, PLC/PKC and the influx of extracellular calcium through the VSCC) is essential to mediate the stimulatory effects of ghrelin on GH release. Lack of functioning of any one of the above mentioned pathways will lead to the cessation of ghrelin's stimulatory effects on GH release.

6.1.2. Food intake

Several studies have demonstrated the potent orexigenic actions of ghrelin in mammals (Tschop et al., 2000, Wren et al., 2001a, Wren et al., 2001b, Asakawa et al., 2001a, Date et al., 2001, Tolle et al., 2002, Lawrence et al., 2002). Subcutaneous administration of rat ghrelin for two weeks increased food intake and weight gain in mice (Tschop et al., 2000). Tschop et al. (2000) also found that a single subcutaneous administration of ghrelin caused an increase in the respiratory quotient (RQ), reflecting an increase in the utilization of carbohydrates and reduction in fat utilization. In order to study the role of GH in mediating the ghrelin-induced

positive energy balance and adiposity, Tschop et al. (2000) injected GH into wild type mice but found no change in the RQ, suggesting that the positive energy balance induced by ghrelin is independent of GH. Further, in GH-deficient mice, ghrelin induced a positive energy balance and a tendency to eat. To study the role of NPY in mediating ghrelin induced food intake and adiposity, Tschop et al. (2000) injected ghrelin into NPY-deficient mice. A significant increase in food intake and adiposity was observed, suggesting that NPY is not essential in mediating the orexigenic effects of ghrelin. Wren et al. (2001a) found that intravenous infusion of ghrelin into healthy humans increased appetite and food intake without affecting gastric emptying. Among the various nuclei into which ghrelin was administered, only microinjections in to the arcuate nuclei and paraventricular nuclei (PVN) increased food intake, with the former one being the most responsive (Wren et al., 2001b). Wren et al. (2001b) also found an increase in appetite and food intake after intraperitoneal injections of ghrelin. ICV administration of ghrelin increased food intake in a dose dependent manner in satiated and freely fed rats (Date et al., 2001). ICV administration of ghrelin in spontaneous dwarf rats (SDR, rats lacking GH gene) stimulated food intake, suggesting that ghrelin-induced food intake is independent of GH (Date et al., 2001). In rats, ghrelin administration increased *c-fos* mRNA expression in the dentate gyrus and hippocampus, where the GHS-R mRNA is abundant (Date et al., 2001). Asakawa et al. (2001a) found that ICV administration of ghrelin stimulate gastric emptying, food intake and a positive

energy balance. They also showed that NPY receptor antagonists blocked ghrelin-induced feeding and IP injections of ghrelin stimulated food intake.

Leptin is an adipocyte derived satiety hormone. Ghrelin stimulates *c-fos* expression in the leptin – responsive neurons in the arcuate nucleus (Traebert et al., 2002). Ghrelin electrical activity in 76% of cells in the arcuate nuclei is inhibited by leptin. These data suggest that ghrelin's orexigenic action may be mediated by its interaction with the leptin-sensitive neurons in the arcuate nuclei. Toshinai et al. (2001) found that coadministration of ghrelin decreased the suppressive effects of leptin on food intake. They also found that the coinjection of leptin attenuated the orexigenic actions of ghrelin. Shintani et al. (2001), demonstrated that ghrelin antagonizes the action of leptin through the activation of NPY neurons. ICV administration of ghrelin increased the expression of NPY mRNA and food intake. Coinjection of NPY Y/Y1 receptor antagonists with ghrelin blocked this effect, suggesting a role for NPY in mediating the orexigenic action of ghrelin. The leptin-induced reduction in NPY mRNA expression was abolished by the administration of ghrelin. These data further suggest the role of NPY in mediating ghrelin-induced orexia. Kamegai et al. (2001) found an increase in NPY and AGRP mRNA expression in the arcuate nuclei and an increase in bodyweight after chronic infusion of ghrelin into the rat brain. This evidence suggests that NPY/AGRP neurons are also targets of ghrelin action in regulating food intake. ICV administration of ghrelin increases the AGRP mRNA expression in the rat arcuate nuceli (Kamegai et al., 2000). Antibodies and antagonists of AGRP decreased ghrelin induced food intake

in rats (Nakazato et al., 2000). Most recently, Seoane et al. (2003) demonstrated that AGRP and NPY neurons in the arcuate nuclei are targets of ghrelin action. Of the four appetite regulatory peptide mRNAs (AGRP, melanin concentrating hormone [MCH], preproorexin and NPY) studied following the ICV administration of ghrelin in rats, only AGRP and NPY mRNAs in the arcuate nuclei showed significant increases in expression. Further evidence for the role of arcuate nuclei neurons in mediating the orexigenic actions of ghrelin came from the studies of Shiiya et al. (2002), who found that ICV administration of ghrelin failed to stimulate food intake in monosodium glutamate (a chemical that ablates the arcuate nuclei) treated rats. Together, these studies suggest that ghrelin acts on the NPY/AGRP and POMC neurons in the arcuate nuclei to facilitate food intake.

A two fold increase in preprandial plasma ghrelin levels and postprandial normalization after 1 hour in humans suggests that ghrelin may act as a meal initiator (Cummings et al., 2001, Shiiya et al., 2002). Shiiya et al. (2002) found a diurnal pattern in circulating plasma ghrelin levels with the maximum levels at 2 AM. Oral and intraventricular administration of glucose decreased plasma ghrelin levels. However, the plasma ghrelin levels in obese humans were found to be lower than in lean humans (Tschop et al., 2001). Plasma ghrelin levels were also found to be lower in *ob/ob* (lacking leptin) and *db/db* (lacking leptin receptor) mice as well as in Zucker fatty (*fa/fa,* lacking leptin receptor) rats (Ariyasu et al., 2002). This decrease in the ghrelin levels may be due to malfunctioning in the leptin system or the increased insulin level that is commonly associated with obesity. Leptin transgenic

(skinny) mice showed a tripling in plasma ghrelin levels (Ariyasu et al., 2002). Ukkola et al. (2001) found a mutation in ghrelin gene in obese patients that might cause a malfunction in ghrelin synthesis.

The role of ghrelin in macronutrient preference is not well studied. Recently, Beck et al. (2002) found an increase in plasma ghrelin levels associated with the intake of a high carbohydrate meal and a reduction in plasma ghrelin levels following a high fat meal. These results suggest that ghrelin secretion is sensitive to diet composition and the decrease in plasma ghrelin levels after a high fat meal may act as a mechanism that signals anorexia, thereby preventing diet-induced obesity.

Plasma ghrelin levels showed an inverse relationship with the anorexigenic peptide glucagon like peptide (GLP-1) (Djurhuus et al., 2002). GLP-1 levels increased dramatically after an oral glucose tolerance test, while there was a significant reduction in plasma ghrelin levels (Djurhuus et al., 2002). These results suggest a postprandial ghrelin - GLP-1 interplay to reduce food intake.

The possible role of the vagus nerve in mediating the effects of ghrelin on food intake has been studied. In order to identify whether ghrelin's effects on food intake is effected by a vagally mediated pathway, Asakawa et al. (2001a) injected ghrelin intraperitoneally into vagotomized mice. The results of this experiment suggest that vagotomy prevents the stimulatory effects of ghrelin on food intake and the ghrelin induced increase in NPY mRNA expression. Ghrelin also decreases the afferent activity of the gastric vagal nerve, thereby negating the anorexigenic action of cholecystokinin (CCK) mediated by the activation of vagal afferent activity.

In a nutshell, the above literature survey implies that ghrelin stimulates food intake in mammals and this effect appears to be mediated through the interactions of ghrelin with the NPY/AGRP system, leptin and GLP-1. Ghrelin might also effect its actions through the vagus nerve.

6.1.3. Other functions

Studies have been conducted in several species to identify the various other biological functions of ghrelin. Extensive studies by Date et al. (2002) found the presence of ghrelin in the alpha cells of human and rat pancreas, suggesting an endocrine or autocrine role of ghrelin in the regulation of insulin secretion from the pancreatic beta cells. Ghrelin stimulated insulin secretion from isolated rat pancreatic beta cells and increased cytosolic free Ca^{2+} concentration in beta cells (Date et al., 2002). Murata et al. (2002) found that exposure of hepatoma cells, which have GHS-R, to ghrelin induces several insulin induced activities such as tyrosine phosphorylation of insulin receptor substrate (IRS-1), association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, mitogen-activated protein kinase activity and cell proliferation. They found that, unlike insulin, ghrelin increased gluconeogenesis. These results suggest that ghrelin may modulate insulin activity in humans.

The role of ghrelin in reproduction is poorly understood. Ghrelin did not change luteinzing hormone (LH) or follicle stimulating hormone (FSH) secretion from dispersed rat pituitary cells or *in vivo* (Kojima et al., 1999). Furuta et al. (2001) found that ICV administration of ghrelin suppresses LH pulse frequency and LH

concentration in ovariectomized rats. Ghrelin immunoreactivity has been detected in the testis and ovary of rats (Caminos et al., 2003). Ghrelin immunoreactivity has been detected in the ovary of rats. Ghrelin levels in the polycystic ovarian syndrome (PCOS) women were found to be significantly lower than normal, levels and it was correlated with degree of insulin resistance (Schofl et al., 2002).

Ghrelin has a vasodilatory effect in humans as evidenced by the increase in forearm blood flow following an intra-arterial ghrelin infusion (Okumura et al., 2002). Both ghrelin and des-acyl ghrelin inhibited apoptosis in cardiomyocytes and endothelial cells (Baldazani et al., 2002). This study gives further evidence for the cardioprotective effect of ghrelin. Intravenous bolus injection of ghrelin decreased mean arterial pressure (hypotensive action), and increased the cardiac index and stroke volume index (Nagaya et al., 2001a, 2001b). IV infusion of ghrelin did not change urine volume, urinary sodium excretion and urinary creatinine secretion (Nagaya et al., 2001b).

Sibila et al. (2002) found that ghrelin has a gastroprotective effect against ethanol-induced gastric ulcers in rats. They found that the gastroprotective effect of ghrelin is mediated by the release of nitric oxide (NO). Additionally, Sibila et al. (2002) demonstrated that the central effects of ghrelin in gastroprotection are mediated by the sensory nerves and not by the vagus nerve. ICV and IP administrations of ghrelin induced anxiogenic activities in rats, increased hypothalamic CRF mRNA levels and plasma corticosterone levels, suggesting a role for ghrelin in mediating stress responses (Asakawa et al., 2001b). Trudel et al.

(2002) found that ghrelin is a potent gastroprokinetic agent in rats and it increases gastric emptying. ICV and IP administration of ghrelin stimulated vasopressin release in a dose dependent manner in conscious rats. Pretreatment of rats with an antiserum for NPY reduced the plasma AVP concentration induced by the ICV administration of ghrelin, suggesting a role for NPY in mediating the stimulatory role of ghrelin on AVP release (Ishizaki et al., 2002). Ghrelin is also involved in sleep in humans. Weikel et al. (2002) demonstrated that intravenous administration of ghrelin increases slow wave sleep in humans.

7. Factors regulating ghrelin secretion

The control of ghrelin secretion is not well understood. The first study to report the effects of a hormone in modifying ghrelin levels was conducted by Mustonen et al. (2001), who found that exogenous melatonin administration reduced plasma ghrelin levels. Preprandial and postprandial physiological and neuroendocrinological changes such as the postprandial increase in glucose and insulin may act as cues that regulate ghrelin secretion.

8. Ghrelin in non-mammalian vertebrates

Ghrelin in non-mammalian vertebrates is an emerging area of interest in comparative endocrinology. Most of the studies have been conducted in birds, amphibians and fish.

8.1. Avians

Ahmed and Harvey (2002) using immunohistochemistry demonstrated the wide distribution of ghrelin in the chicken brain. They also found that intravenous

injections of mammalian ghrelin (10 microgram/kg) stimulate GH secretion in chicken. Recently, Kaiya et al. (2002) reported the cDNA sequence and peptide structure of ghrelin from the stomach of chicken, *Gallus gallus*. Chicken ghrelin is 26 amino acids in length and the mature peptide has 54% sequence similarity with human ghrelin. The third serine in the mature peptide is conserved in chicken ghrelin. Chicken ghrelin occurs as n-*octanoyl* ghrelin or n-*decanoyl* ghrelin. Although, wide distribution of ghrelin mRNA has been detected in the brain, lung and intestine using RT-PCR analysis. Northern blot analysis detected ghrelin mRNA only in the stomach of chicken. In chicken, intravenous injection of chicken ghrelin increased plasma GH and plasma corticosterone levels (Kaiya et al., 2002). ICV administration of ghrelin decreased food intake in chicken (Saito et al., 2002). These results suggest that unlike in mammals, ghrelin is an anorexigen in chicken.

8.2. Amphibians

cDNA encoding ghrelin has been identified from bullfrog, *Rana catesbeiana* (Kaiya et al., 2001). Bullfrog has three forms of ghrelin, a 28 amino acid n-octanoylated ghrelin and the 27 amino acid ghrelin with either the n-octanoyl or the n-decanoyl modification. The important structural difference of bullfrog ghrelin with ghrelin from other species is the presence of a "GLTFSLP" in the N-terminal region. The octanoyl modification is in the third threonine instead of the serine as observed in other species. Bullfrog ghrelin mRNA is present in the stomach (by Northern blot analysis), brain, small intestine, pancreas, testes, heart, lung and gall bladder (by RT-PCR analysis). Both frog ghrelin-27 and frog ghrelin-28 increased

Ca^{2+} concentration in CHO cells expressing GHS-R. Frog ghrelin-27 simulated GH and PRL release from dispersed pituitary cells of bullfrog. More recently, ghrelin has also been purified from the brain and stomach of the frog, *Rana esculenta* (Galas et al., 2002).

8.3. Fish

Recently, the structure of ghrelin cDNA and peptide has been reported from eel (Kaiya et al., 2003a), tilapia (Kaiya et al., 2003b, Parhar et al., 2003) and the rainbow trout (Kaiya et al., 2003c). A limited number of studies reported evidence for the presence and biological actions of ghrelin in fish. A ghrelin like peptide was detected in the plasma of spawning burbot, *Lota lota* (Mustonen et al., 2001). Rat ghrelin induces GH secretion *in vitro* from incubated whole pituitaries of tilapia (Riley et al., 2002). Kozaka et al. (2003) demonstrated that the intracranial administration of ghrelin inhibited water intake in the seawater eel. In order to avoid redundancy, the details of relevant reports on fish ghrelin will be discussed in various data chapters and in the general discussion.

9. Hypotheses

This project is based on two main hypotheses. They are: (i) ghrelin is present in the goldfish, and (ii) ghrelin is involved in the regulation of food intake and pituitary hormone secretion in the goldfish.

10. Specific objectives of the project

The objectives of this project are to (i) identify the structure of the gene encoding ghrelin in the goldfish, (ii) identify the ghrelin mRNA expressing regions

in goldfish and detect the meal related changes in ghrelin mRNA expression and serum levels, and (iii) to study the biological actions of ghrelin in goldfish using native peptides.

Reverse transcription (RT) – Polymerase chain reaction (PCR) and 5'/3' rapid amplification of cDNA ends (RACE) will be conducted to identify the complete cDNA sequence of ghrelin in goldfish. Genomic DNA PCR will be used to identify the structure of ghrelin gene in goldfish. Based on the ghrelin nucleotide sequence information, probes will be developed to identify the expression of ghrelin mRNA in the goldfish brain and peripheral tissues. A variety of techniques including Northern blot and slot blot analyses and in situ hybridization will be used to detect ghrelin mRNA expression and profile the meal related changes in its mRNA expression in the goldfish. Once the gene encoding ghrelin has been identified, the mature peptide structure will be identified using bioinformatic tools. Based on this sequence information, native goldfish ghrelin peptide will be synthesized. This peptide will be used for studying the biological actions of ghrelin in goldfish. I am specifically interested in studying, (I) the role of ghrelin in the regulation of GH release from the pituitary of goldfish, and (II) its role in the regulation of food intake in goldfish. *In vitro* and *in vivo* studies will be conducted to determine the role of ghrelin in the regulation of GH secretion in goldfish. ICV and IP injections will be conducted and food intake will be quantified to determine the central and peripheral effects of ghrelin on food intake. A radioimmunoassay (RIA) for the active form of ghrelin will be validated to measure the meal-related

changes in serum ghrelin levels. Using a variety of molecular biology and *in vitro* and *in vivo* (whole animal) physiology tools, this project will report the gene organization, peptide structure, distribution of the mRNA encoding preproghrelin, and its role in regulating the pituitary hormone secretion and food intake in goldfish.

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SECTION B - GALANIN

1.1. Overview

Galanin is a 29 amino acid (30 amino acids in humans) peptide, first identified from the porcine intestine (Tatemoto et al., 1983). The name galanin is based on the terminal amino acids in the peptide; glycine in the amino terminal and alanine in the carboxy terminal. Galanin has been identified from a number of mammalian and non-mammalian species (Iismaa and Shine, 1999). Galanin is involved in the regulation of various physiological functions including feeding, reproduction, growth, regulation of hormonal release from the pituitary gland, memory and cognition, pain and nerve regeneration, cardiovascular functioning and mobility of gastric sphincters (Iismaa and Shine, 1999). The aim of this section is to give an overview of the structure, distribution and some physiological functions of galanin. In depth discussion of relevant topics will be conducted in the data chapters and general discussion.

2. Structure of galanin

2.1. Gene

The structure of galanin gene has been identified from many mammals including humans (Evans et al., 1993) and mice (Kofler et al., 1996). The galanin gene structure is highly conserved among mammalian species. Human (Evans et al., 1993) and mouse (Kofler et al., 1996) galanin have identical intron:exon organization and are both comprised of six exons and five introns. The exon 1 is non-coding and the exons 2-5 constitute the coding region. The stop codon is

present in exon 6. Preprogalanin mRNA is comprised of distinct regions encoding for the signal peptide region, mature peptide region and the galanin message associated peptide (GMAP) region (Kofler et al., 1996). The site of human galanin gene is the chromosome 11q13.3-13.5 (Iisma and Shine., 1999). Preprogalanin cDNAs have been identified from a number of other mammalian vertebrates including the pig (Rokaeus and Brownstein, 1986), sheep (Sillard et al., 1991), rat (Kaplan et al., 1988) and cow (Rokaeus and Carlquist, 1988). Recently, the cDNAs encoding two forms of preprogalanin were identified in the quail brain (Kohchi and Tsutsui, 2000). The intron:exon organization of galanin gene in non-mammalian vertebrates is currently not known.

2.2. Peptide

2.2.1. Mammals

In mammals, galanin is derived from a 123 amino acid precursor peptide, the preprogalanin, along with a 59 amino acid peptide known as the GMAP (Kofler et al., 1996). 1-13 amino acids in the N-terminal region of the mature peptide is 100% conserved in all mammals from which galanin has been identified. Lindskog et al. (1992) performed the secondary structure prediction analysis of the primary sequences of six galanin peptides. The N-terminal portion (1-13 amino acids) is featured by the presence of a beta-sheet. This model also predicts alpha-helix conformations in the N-terminal region of the pig, rat and sheep galanin. Human and bovine galanin has alpha helices in the regions between positions 13 and 20. The

human and bovine peptides were named as galanin A and the pig, rat and sheep galanin lacking alpha-helices were termed galanin B (Lindskog et al., 1992).

2.2.2. Non-mammalian vertebrates

Quail preprogalanin normal form has a 29 amino acid galanin mature peptide, while the alternate transcript has an insert of 69 nucleotides in the mature peptide region, resulting in a 59 amino acid galanin peptide (Kohchi and Tsutsui, 2000). In both forms of quail galanin, the N-terminal 1-13 amino acids are identical. Galanin is a 29 amino acid peptide in the tortoise (Wang et al., 1999a) and alligator (Wang and Conlon, 1994a). A 29 amino acid galanin peptide has been purified from the trout (Anglade et al., 1994), tuna fish (Habu et al., 1994), bowfin (Wang and Conlon, 1994b), dogfish (Wang and Conlon, 1994b) and sturgeon (Wang et al., 1999b). The N-terminal 1-13 amino acids of the galanin peptide are conserved in all fish species from which galanin has been identified, except in the tunafish where the serine (S) at position 6 has been substituted by alanine (A) (Habu et al., 1994).

3. Distribution of galanin

3.1. mRNA

Galanin mRNA is widely expressed in mammals. In mouse, galanin mRNA expressing cells are present in the arcuate nuclei, paraventricular nuclei, periventricular nuclei and the dorsomedial nucleus of the hypothalamus (Cheung et al., 2001). Galanin mRNA expression is found in the paraventricular, arcuate and dosromedial nuclei and the lateral hypothalamus of rats (Pu et al., 1999). Galanin mRNA is highly expressed in the somatotropes and lactotropes of rats (Hsu et al.,

1991). Northern blot analysis detected galanin mRNA expression in peripheral tissues including the heart, lungs, gastrointestinal tract, liver, pancreas, thyroid and adrenal gland (Rokaeus, 1994). *In situ* hybridization and autoradiography studies on the distribution of preprogalanin mRNA in rat magnocellular neurons revealed preprogalanin mRNA associated with rough endoplasmic reticulum, in perikarya, dendrites and axons (Landry and Hokfelt, 1998). The wide expression of galanin mRNA in the brain and pituitary indicates a potential role for galanin in the regulation of feeding in mammals. In quail, the preprogalanin mRNA encoding the short galanin peptide is expressed in the brain and intestine. The preprogalanin mRNA encoding the long form was only expressed in the ovary of quail (Kohchi and Tsutsui, 2000).

3.2. Peptide

3.2.2. Mammals

Galanin like immunoreactivity is widely present in the brain of many mammals. Using a porcine antibody, wide distribution of galanin like immunoreactivity had been identified in the rat brain. High concentrations of galanin-ir were detected in brain regions including the hippocampus, amygdala, hypothalamus, nucleus tractus solitarius and the trigeminal nucleus (Skofitsch and Jacobowitz., 1989). Moderate levels of galanin-ir was found in the cerebrum and cerebellum. Melander et al. (1986) also found galanin like immunoreactivty in cell bodies of hypothalamic and thalamic nuclei. Galanin like immunoreactivity has been identified in monkey gonadotropes (Niimi et al., 1993).

3.2. In fish

Several studies have identified galanin like immunoreactivity in the brain and pituitary of teleost fish (Jadhao and Pinelli, 2001), including the goldfish (Rao et al., 1996). Rao et al. (1996) found a sexual dimorphism in galanin like immunoreactivity in the brain and pituitary of goldfish, using antiserum generated against porcine galanin. Male goldfish has extensive galanin like immunoreactivity in the olfactory bulbs, telencephalon, infundibulum, mesencephalic tegmentum, optic tectum and medulla oblongata, while female goldfish had less galanin immunoreactivity in these regions. Male goldfish have many galanin like immunoreactive preikarya in the nucleus preopticus pars parvocellularis (NPOpp) and isolated galanin like immunoreactive perikarya in the NPO pars magnocellularis and lateral to the NPO. In females, galanin like immunoreactivity was not found in this region. On the other hand, males revealed fewer galanin like immunoreactive neurons than females in the nucleus posterior tuberis. Galanin immunoreactive fibres ramify and terminate among the pars distalis cells of the pituitary gland. Recently, Farrel et al. (2002) identified a plexus of galanin immunoreactive fibres in the vagal lobe of goldfish brain. Rao et al. (1996) found that a small percentage of growth hormone secreting cells colocalize galanin in the goldfish pituitary. Holmqvist and Ekstrom (1991) studied galanin distribution and relation to substance P, vasotocin, and isotocin in the Atlantic salmon (*Salmo salar*). No coexistence of galanin immunoreactive neurons with arginine vasotocin or isotocin was found in the neurosecretory neurons of salmon, as shown with the mammalian counterparts

vasopressin and oxytocin. The wide distribution of galanin in the brain and pituitary indicate that, as in mammals, galanin may be a physiological regulator in fish.

4. Galanin binding sites in fish

Moons et al. (1991) conducted autoradiographic studies on the distribution of galanin binding sites in the brain and pituitary of sea bass, *Dicentrarchus labrax*. Galanin binding sites were present in all parts of the dorsal and ventral telencephalon, hypothalamus, thalamus, optic tectum, ventral medulla oblongata and in the pituitary. Dense galanin binding sites were confined to the area occupied by the prolactin cells in the rostral part of the adenohypophysis. In the pituitary gland, galanin-like immunopositive fibres were found in close association with adrenocoticotropin-prolactin- thyroid stimulating hormone-gonadotropin- and growth hormone-producing cells (Moons et al., 1991). Using a radioligand binding assay, the presence of galanin binding sites has been demonstrated in the cellular regions, fiber tracts and in neuropil areas throughout brain except the olfactory bulb, pineal organ and cerebellum of Atlantic salmon, *Salmo salar*. Galanin binding sites were also found in the pituitary, telencephalon, dorsolateral thalamic nuclei and raphe nuclei and in association with the forebrain bundles of Atlantic salmon (Holmqvist and Carlberg, 1992).

5. Galanin receptors in mammals

cDNAs encoding three galanin receptors, galanin R1 (GAL-R1), R2 (GAL-R2) and R3 (GAL-R3) were identified in mammals (Depcynszki et al., 1998). Galanin receptors belong to the G-protein coupled receptor family (GPCR) family

(Depcynzki et al., 1998). In mammals, galanin R1 mRNA was found in high concentrations in ventral hippocampus, thalamic nuclei, amygdala, brain stem nuclei, hypothalamus and posterior horns of the spinal cord (Depcynszki et al., 1998). Galanin R2 mRNA is expressed in brain areas including the major hypothalamic nuclei, hippocampus, entorhinal and piriform cortex, amygdala and the pituitary gland (Fathi et al., 1997). Galanin R2 expression was found in the lactotropes, somatotropes, gonadotropes and thyrotropes, suggesting that this receptor plays a major functional role in pituitary hormone secretion (Depczynski et al., 1998). Localization of galanin R3 mRNA showed that the galanin R3 transcript is widely distributed, but expressed in low abundance with highest levels in the pituitary and hypothalamus (Smith et al., 1998). Galanin R2 shares less than 40% overall sequence identity with galanin R1. There are no reports available on the structure and functions of galanin receptors in non-mammalian vertebrates.

6. Biological actions of galanin

Galanin is involved in the regulation of many physiological functions in mammals and non-mammalian vertebrates. In mammals, galanin has both stimulatory (e.g. LH release from pituitary cells) and inhibitory functions (eg: neurotransmitter secretion). The objective of this section is to give a brief overview of galanin's role in the regulation of food intake and pituitary hormone secretion in mammals and non-mammalian vertebrates.

6.1. Regulation of food intake

6.1.1. In mammals

Galanin is an orexigen in mammals. In rats, injections of galanin into the hypothalamus stimulate feeding behavior (Crawley et al., 1990). However, continuous infusion of galanin was ineffective in increasing food intake and body weight gain (Smith et al., 1994). In rats, acute injections of galanin into the paraventricular nucleus produce a shift in metabolism towards a state of energy conservation characterized by reduced thermogenesis. Galanin antagonist or antisense oligonucleotide injection into the hypothalamus of rats caused a reduction in galanin levels and suppression of fat intake (Akabayashi et al., 1994a). In rats, a small increase in galanin levels in the lateral portions of the posterior paraventricular nucleus was observed 3h into the dark phase, suggesting that galanin is involved in the regulation of food intake (Akabayashi et al., 1994b). Galanin has specific stimulatory effects in the consumption of fat and carbohydrate (Chae et al., 1995). In rats, central administration of galanin into the hypothalamus increased fat and carbohydrate ingestion, while no effects were found on protein uptake. Infusion of galanin antisense oligonucleotides in the paraventricular nucleus inhibited feeding. These results indicate that galanin is involved in the regulation of food intake in mammals.

The galanin antagonist, galantide, attenuated NPY-induced LH release, indicating the role of galanin in the release of NPY (Kalra, 1996). Recently, Bergonzelli et al., (2001) found that galanin is a potent stimulator of NPY release

from the perifused rat primary hypothalamic neurons. These results suggest that NPY is involved in mediating the orexigenic actions of galanin in mammals. Sahu (1998a,b) demonstrated that brain injections of leptin decrease hypothalamic galanin, melanin concentrating hormone (MCH), proopiomelanocortin (POMC) and NPY gene expression in ad-libitum fed rats. These results suggest that the role of leptin in the regulation of feeding is not restricted to its action on NPY but also mediated by the hypothalamic galanin, MCH and POMC neurons. Opiate receptor antagonist, naloxane reduces galanin-induced feeding in a dose-dependent fashion (Sahu et al., 1995). Insulin has a strong inhibitory effect on galanin gene expression and peptide concentration. Micropunch experiments of paraventricular nucleus show a significant decrease in galanin mRNA expression after subcutaneous administration of insulin in rats (Tang et al., 1997). These results indicate that anorexigens, such as leptin and insulin, inhibit galanin synthesis and release as part of the satiety mechanisms in mammals.

6.1.2. In non-mammalian vertebrates

Among non-mammalian vertebrates, most of the studies on the appetite regulatory functions of galanin have been conducted in goldfish. In goldfish, ICV injections of galanin were found to stimulate food intake (de Pedro et al., 1995). Galanin-induced feeding was observed only during the first 2 hour after the injection, but not during 2-8 hour post-injection. Peripheral injections of galanin were ineffective in stimulating food intake in goldfish. These results show that galanin is involved in the central regulation of feeding in fish (de Pedro et al., 1995). De Pedro

et al. (1995) also found that pretreatment with yohimbine, an alpha-2-adrenergic receptor antagonist, blocked the orexigenic effects of galanin, suggesting that galanin-induced feeding is mediated through the alpha adrenergic system. Galanin stimulated food intake in goldfish pretreated with prasozin, an alpha-1-adrenergic receptor antagonist. A galanin receptor antagonist, galantide, also blocked the stimulatory effects of galanin on food intake (de Pedro et al., 1995). These results suggest that galanin acts in the brain to stimulate food intake in goldfish.

Recently, Volkoff and Peter (2000) found that ICV administration of galanin stimulates food intake in a dose dependent manner in goldfish. Co-administration of galanin receptor antagonist, M40 blocked the stimulatory effects of galanin on food intake in goldfish (Volkoff and Peter, 2000). These results provide further evidence for the orexigenic actions of galanin in goldfish. The galanin receptor blocker M-40, had no effects on orexin induced feeding in goldfish. However, coinjection of M-40 and NPY resulted in a decrease in food intake suggesting that NPY might play a role in mediating galanin's orexigenic action in goldfish. Coinjection of galanin and BIBP3226, the NPY-Y1 receptor antagonist, blocked the orexigenic effects of galanin. Orexin receptor desensitization using a high dose of orexin A, resulted in the attenuation of NPY and galanin induced food intake. NPY receptor blockade or orexin receptor desensitization resulted in the attenuation of orexigenic actions of galanin, suggesting that the orexigenic action of galanin in goldfish is mediated through the NPY and orexin system.

The only other study on the role of galanin in food intake in non-mammalian vertebrates was conducted in chicken. In neonatal chicken, brain injections of human galanin has no effects on food intake (Ando et al., 2000).

6.2. Regulation of pituitary hormone secretion

6.2.1. GH

6.2.1.b. In mammals

Galanin stimulates GH release *in vitro* and *in vivo* in mammals. Galanin stimulates GH release from dispersed pituitary cells of rats (Baranowska et al., 1999) and perifused pituitary of calves (Barrata et al., 1997). These results indicate that galanin acts directly on the somatotropes to stimulate GH release in mammals. Intravenous injection of human galanin stimulated basal and GHRH induced GH release in humans (Todd et al., 2000). Leibowitz (1998) demonstrated that certain galanin cell groups in the arcuate nuclei coexist with GHRH cells. Galanin from GHRH neurons may act either within the brain as a neurotransmitter or as hypophysiotropic factor at the anterior pituitary. In 72h food-deprived rats, hypothalamic galanin mRNA levels decreased 5.6 fold from control animals, while pituitary galanin in food-deprived animals decreased 22.5-fold compared to control animals (Brogan et al., 1997). The considerable decrease in both hypothalamic and pituitary galanin mRNA may play a significant role in the decreased GH concentrations seen with food deprivation in rats (Brogan et al., 1997). Galanin synthesized in GHRH neurons is likely to be involved in the regulation of GH secretion. Galanin accentuates the pulsatile nature of GH secretion by inhibiting SS

release, and thereby reducing SS inhibition of GH and GHRH release during pulse generation. Brogan et al. (1997) demonstrated that galanin infusion, either through the central or peripheral routes induces GH secretion. They also found that GH regulates galanin expression in the GHRH neurons. In adult male rats, testosterone increases both galanin mRNA expression in the GHRH neurons and also the amplitude of GH pulses (Chan et al., 1996). It is clear in mammals, that galanin stimulates GH release by acting directly on the pituitary somatotropes. Galanin also induces GHRH release and inhibits SS release from the mammalian brain resulting in increased GH secretion.

6.2.1.a. In fish

To the best of my knowledge, there is only one published report that demonstrates a stimulatory role for galanin on GH secretion in fish. Diez et al. (1992) found that intraperitoneal injection of galanin stimulates GH secretion in *Oncorhynchus kistuch*. In goldfish, galanin stimulates GH secretion from pituitary fragments *in vitro* [Komourdijian et al., as cited in Rao et al. (1996); Peng, (1993)]. These results suggest that galanin is a GH releasing peptide in fish.

6.2.2. LH

6.2.1. In mammals

Galanin stimulates LH release *in vitro* and *in vivo* in mammals. Galanin stimulates LH release from the dispersed pituitary cells of rats (Baranowska et al., 1999) and from perifused pituitary fragments of calves (Barrata et al., 1997). These

results indicate that galanin acts directly on the pituitary to stimulate LH release in mammals.

Galanin also stimulates GnRH release thereby regulating LH release. Intra- and inter- cerebroventricular galanin administration in rats stimulates LH release (Cheung et al., 1996). Sahu et al. (1987) found that galanin infusion into the cerebral ventricles of rat evoked the release of LH. Sahu et al. (1987,1994) also found that the induction of LH release by intraventricular infusions of galanin can be blocked by prior treatment with a galanin receptor antagonist, galantide. Arvat et al. (1995) found that within the hypothalamus there is a set of GnRH neurons that produces galanin. Galanin is secreted into the portal circulation in a pulsatile manner and the pulses appear to precede or to be coincident with GnRH pulses (Lopez et al., 1990). It is suggested that galanin is released into the portal vessels from hypophysiotropic neurons and is transported to target cells in the pituitary, where it stimulates the release of LH (Sahu et al., 1994). Galanin, when applied to the arcuate nuclei-median eminence fragment stimulates GnRH release (Lopez et al., 1990). Rossmanith et al. (1996) showed that when the steroid dependent-activation of GnRH neurons and the LH surge which follows are inhibited by either alpha-adrenergic or N-methyl-D-aspartate (NMDA) receptors, the associated induction of galanin mRNA in GnRH neurons is completely blocked. These data suggest that the galanin gene in GnRH neurons is highly related to the activation of GnRH neurons and is tightly coupled to the occurrence of a LH surge. A recent study (Splett et al., 2003) demonstrated that galanin stimulates GnRH induced LH release in rats and

this effect is possible only in the presence of estrogen. The results of the above described studies in mammals, indicate that galanin, in addition to its direct actions on the gonadotropes to stimulate LH release, also stimulates GnRH release, that latter results in an increased LH secretion.

6.2.2. In non-mammalian vertebrates

A preliminary study conducted in our laboratory found that galanin stimulates LH release from the perifused pituitary fragments of goldfish (Peng, 1993). These results suggest that galanin acts directly on the gonadotropes to stimulate LH release in goldfish. However, there are no published results on the effects of galanin on LH release in non-mammalian vertebrates.

7. Hypotheses

Based on the knowledge from the literature available on galanin, I hypothesize that (i) galanin is present in the goldfish, (ii) galanin mRNA is expressed in the brain and periperhal tissues of goldfish, and (iii) there is a meal-related change in galanin mRNA expression in the goldfish brain.

8. Specific objectives of this research project

The specific objectives of this research project are to identify the (i) structure of the cDNA encoding galanin in goldfish, (ii) distribution of galanin mRNA in the brain and peripheral tissues, and (iii) to study the meal-related and starvation-induced changes in galanin mRNA expression in the goldfish. I will use the techniques listed in section A, to achieve these goals. To the best of my knowledge, this study will be the first to identify the galanin cDNA structure and distribution in a

teleost fish. Characterization of the cDNA sequence encoding galanin will help to deduce the structure of galanin peptide. It will also enable me to design specific probes useful for the Northern blot analysis of galanin mRNA in the goldfish brain and peripheral tissues, and the *in situ* localization of galanin mRNA in the brain nuclei of goldfish. Demonstration of galanin mRNA, in brain regions and specific nuclei that are thought to be involved in the regulation of hunger and appetite, will provide supportive evidence for the orexigenic role of galanin in goldfish. Once the brain regions and nuclei that express galanin has been demonstrated, changes in galanin mRNA expression in these brain regions of goldfish during the scheduled mealtime can be studied. A meal-related change in galanin mRNA expression would add supportive evidence for the orexigenic role of endogenous galanin on appetite regulation in the goldfish.

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CHAPTER 2

GOLDFISH GHRELIN: MOLECULAR CHARACTERIZATION OF THE COMPLEMENTARY DEOXYRIBONUCLEIC ACID, PARTIAL GENE STRUCTURE AND EVIDENCE FOR ITS STIMULATORY ROLE IN FOOD INTAKE

2.1. Introduction

Growth hormone secretagogues (GHS) are synthetic compounds that stimulate growth hormone (GH) secretion (Bowers, 1998) by binding to the growth hormone secretagogue receptor (GHS-R). Ghrelin, the first known endogenous ligand of GHS-R, is an acylated gut/brain peptide originally isolated from the rat stomach (Kojima et al., 1999). The first seven amino acids in the N-terminal region of the ghrelin peptide is conserved in all rats, mouse and humans (Kojima et al., 1999). The first four amino acids "GSSF", with the *n*-octanoyl group in the third residue (serine), is considered to be the "active core" of the peptide (Bednarek et al., 2000). Ghrelin cDNA has been identified from several species including human (Kojima et al., 1999), rat (Kojima et al., 1999), mouse (Kojima et al., 1999), and the bullfrog (Kaiya et al., 2001). Ghrelin gene structure has been characterized in humans (Wajnrajch et al., 2000), rat and mouse (Tanaka et al., 2001). Ghrelin mRNA expression has been detected mainly in the brain and stomach of rat (Kojima et al., 1999) and in the stomach of bullfrog (Kaiya et al., 2001). Recently, ghrelin

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and GHS-R mRNA distribution was demonstrated in human brain, gut and several peripheral tissues including the liver and spleen (Gnanapavan et al., 2002). Ghrelin stimulates GH secretion in mammals (Kojima et al., 1999) and in bullfrog (Kaiya et al., 2001). Ghrelin stimulates food intake in rats (Tschop et al., 2000, Wren et al., 2000).

GHS-R has been identified in pufferfish and it has a high similarity with the mammalian GHS-R (Palyha et al., 2000). The GHS, KP-102 stimulates GH secretion in tilapia (Shepherd et al., 2000). Goldfish is a well characterized model for studying the neuroendocrine regulation of GH secretion and food intake in non-mammalian vertebrates, yet the potentially important regulator of these two parameters, ghrelin, has not been studied in the model system. The aim of this study was to identify the structure of ghrelin cDNA in the goldfish, to detect ghrelin mRNA expression in the brain and peripheral tissues, and to elucidate the role of native ghrelin peptide on food intake.

2.2. Materials and Methods

Animals

Male and female goldfish (*Carassius auratus*) of the common or comet varieties and approximately 40 g body weight, were purchased from the Mount Parnell Fisheries (Marcersberg, PA, USA) and maintained under a simulated natural photoperiod of Edmonton, Alberta, Canada. Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) before decapitation and dissection of the brain and other tissues. All protocols were

approved by the University of Alberta, Department of Biological Sciences Animal Care Committee.

Cloning and sequence analysis

Total RNA was extracted using the Trizol reagent (Invitrogen Life Technologies, Canada) from the goldfish gut and brain. RT and 3' RACE were carried out as described previously (Volkoff and Peter, 2001a). To isolate the 3' end of the goldfish ghrelin cDNA, two degenerate primers, GRN1 (5'GTCAGYGC WGGYTCCAGCTTC3') and GRN2 (5'GGYTCCAGCTTCYTSAGCC3') were designed based on the sequences of mammalian ghrelin and trout ghrelin sequence (Kaiya H and Kangawa K, personal communication). The desired product was purified from the gel, subcloned and sequenced as described earlier (12). 5' RACE was conducted using gene specific primers GRN3 (AATCATTTTAATGGTTTATA 3') and GRN4 (5'GCTGAATCTTGTAGAACTTT3') based on the 3' sequence. PCR for full-length cDNA was conducted with the gene specific primers GRLF (5'CTGTGCATTCTGCATACATATTTGAG3') and GRLR (5'GTTTTGGAAGAT TATTACATC3'). The conditions for PCR were 5 minutes of denaturation at 95 C, followed by 35 cycles of denaturation at 95 C, annealing at 45 C and extension at 73 C. To clone the ghrelin gene, PCR was conducted using 100 ng genomic DNA under the same conditions mentioned above.

RT-PCR, Southern blot and Northern blot analyses

Ghrelin expression in different regions of the brain (olfactory bulbs and tracts, telencephalon, hypothalamus, midbrain and hindbrain), pituitary, gut, liver,

spleen, muscle, gill, heart, kidney, eye, testis and ovary was detected by RT-PCR using the same primers and PCR conditions used for cloning the full length ghrelin cDNA. The reactions were electrophoresed on a 1.5% agarose gel and transferred to a Hybond nylon membrane by capillary transfer. Hybridization with a [α-^{32}P]dCTP labeled probe covering the full length ghrelin cDNA was performed using methods described earlier (Volkoff and Peter, 2001a). Membranes were exposed to a Phosphorscreen (Molecular Dynamics, Sunnyvale, CA) for 1 hour and the screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). PCR for β-actin was conducted as an internal control. For Northern blot, 40μg of total RNA was extracted from various brain regions described above and the gut, electrophoresed on a denaturing agarose gel (1.5%) with formaldehyde, transferred to a membrane and hybridized as described earlier, except in this case the screen was exposed for 7 days. To check the concentration of the RNA used for the Northern blot, membranes were stripped and reprobed with an [α-^{32}P]dCTP labeled partial cDNA probe for goldfish β-actin (unpublished results, GenBank accession number AF079831).

ICV injections and goldfish ghrelin

Goldfish ghrelin (1-19) with an n-octanoyl modification in the third residue (serine) of the mature peptide was synthesized using the solid phase (9-fluorenylmethoxycarbonyl) strategy at the Salk Institute. ICV injections were conducted as described earlier (Volkoff and Peter, 2001a). A 5 μl microsyringe was stereotaxically placed in the posterior preoptic region of the brain and 2 μl of the test

solution [saline, 1 or 5 ng/g body weight (BW) of goldfish ghrelin (1-19)] administered. Following the ICV injection and closure of the head wound, fish were returned to their aquarium and recovered from anesthesia within 5 minutes. Food pellets were administered 15 minutes (including the recovery time) after the ICV injection and cumulative food intake for each fish measured for 1 h.

Statistical analysis

Statistical analysis was conducted using ANOVA followed by Student-Newman-Kuels multiple comparison test. Significance was considered at $P<0.05$. Data are expressed as mean ± SEM.

2.3. Results

A 490 bp preproghrelin cDNA was identified from the gut and the brain (Figure 2.1; GenBank accession number AF454389). The deduced structure of preproghrelin peptide contains 103 amino acids. The signal peptide region is 26 amino acids in length, predicted by the SignalP server (www.cbs.dtu.dk/services/SignalP). The mature peptide region begins immediately after the signal peptide. There are two putative cleavage and amidation signals (GRR) after 12 amino acids and after 19 amino acids. The region after the mature peptide to the stop codon (C-terminal peptide) has 55 amino acids. An analysis of genomic DNA revealed that the goldfish ghrelin gene has three short introns and four exons (sequence not shown, GenBank accession number AF454390, Figure 2.2.). Exon 1 (205 bp) contains the 5' untranslated region (UTR) and the region encoding the first 12 amino acids of mature peptide region. Exon 2 (79 bp) encodes the remaining portions of the mature

peptide and a part of the C- terminal peptide. Exon 3 (109 bp) encodes a part of the C-terminal peptide. Exon 4 (95 bp) encodes the terminal region of the C-terminal peptide and the 3' UTR. The introns are 129 bp (intron 1), 96 bp (intron 2) and 267 bp (intron 3) in length. Figure 2.3 is the comparison of the sequences of mature peptide ghrelin from various species. RT-PCR followed by Southern blot analysis detected ghrelin mRNA expression in the telencephalon, hypothalamus, gut, spleen, and gill (Figure 2.4). Northern blot analysis detected ghrelin mRNA expression only in the gut (Figure 2.5). ICV administration of goldfish ghrelin (1-19) stimulated food intake. A dose of either 1ng/g bodyweight or 5 ng/g bodyweight ghrelin stimulated food intake significantly, compared to the unhandled and saline treated fish (Figure 2.6).

2.4. Discussion

The deduced amino acid sequence of goldfish ghrelin (1-19) has 47%, 36% and 31% identity with the human, rat and bullfrog ghrelin, respectively. The "active core" (GSSF) of ghrelin is conserved in mammalian species whereas in the bullfrog it is "GLTF". In goldfish this region is "GTSF". The substitution of the second amino acid to threonine from serine is due to a single base substitution (AGC to ACC). Both serine and threonine are functionally similar and the substitution likely does not affect the structure and biological function of the goldfish ghrelin. The presence of two cleavage sites and amidation signals suggests the presence of two forms of the peptide, a short form which is 12 amino acids in length and a long form that is 19 amino acids in length. Both peptides retain the "active core" intact.

Similarly, prepro-orexin contains two amidation signals and can be processed into two peptides, orexin-A and orexin-B, that are biologically active (Sakurai et al., 1998). The identity of the *n*-acyl modification in goldfish ghrelin is not known. However, the goldfish ghrelin (1-19) used in this study is n-octanoylated (the most commonly found modification of ghrelin). Bullfrog has both octanoylated and decanoylated ghrelin which are biologically active (Kaiya et al., 2001). Synthetic ghrelins with other acyl modifications are also found biologically active (Matsumoto et al., 2001).

The intron:exon organization of the goldfish ghrelin gene resembles the human ghrelin gene organization. Goldfish ghrelin gene has short introns compared to the introns in mammalian ghrelin genes (Tanaka et al., 2001, Wajnrajch et al., 2000). All intron:exon junctions conform to the GT/AG rule of splicing. Recently, a non-coding 19 bp exon in the 5' UTR of the mouse and rat ghrelin genes was reported. In goldfish, this short exon was not found.

Recently, wide distribution of ghrelin and GHS-R mRNAs in the brain and peripheral tissues has been demonstrated in humans, suggesting the involvement of ghrelin in vital physiological functions (Gnanapavan et al., 2002). From RT-PCR followed by Southern blot analysis, and Northern blot analysis the strongest expression of goldfish preproghrelin mRNA was found in the gut. Similar results have been obtained from rats (Kojima et al., 1999) and the bullfrog (Kaiya et al., 2001). The Northern blots were repeated several times with total RNA and poly(A)$^+$ RNA from the brain regions, but no signal was found. The results of the mRNA

expression studies suggest that gut may be the primary site of ghrelin production in the goldfish. However, the RT-PCR studies show ghrelin expression in the brain. The physiological significance of strong ghrelin mRNA expression in gut, spleen, gill and liver is unknown. Brain ghrelin may be involved in the central regulation of food intake which is indicated by the present results.

A significant increase in food intake after the ICV administration of *n*-octanoylated ghrelin (1-19) suggests that the synthetic peptide is biologically active. It supports the hypothesis that native ghrelin is an orexigenic factor in goldfish. The increase in food intake after ghrelin administration is comparable to the increase in cumulative food intake for 1 hour after the ICV administration of human ghrelin (unpublished observations from our lab). ICV administration of other orexigenic peptides including NPY, orexin, and galanin also stimulates food intake in goldfish (Volkoff and Peter, 2001b). It has been demonstrated that an appetite regulatory mechanism similar to that in mammals exists in fish (Lin et al., 2000).

In summary, this study, for the first time report the ghrelin gene structure and its orexigenic action in a non -mammalian vertebrate. This is the first report of a ghrelin peptide with "GTSFLSP" in the N-terminal region and with two putative cleavage sites and amidation signals. The results presented in this chapter suggest that ghrelin structure and functions might be conserved during vertebrate evolution.

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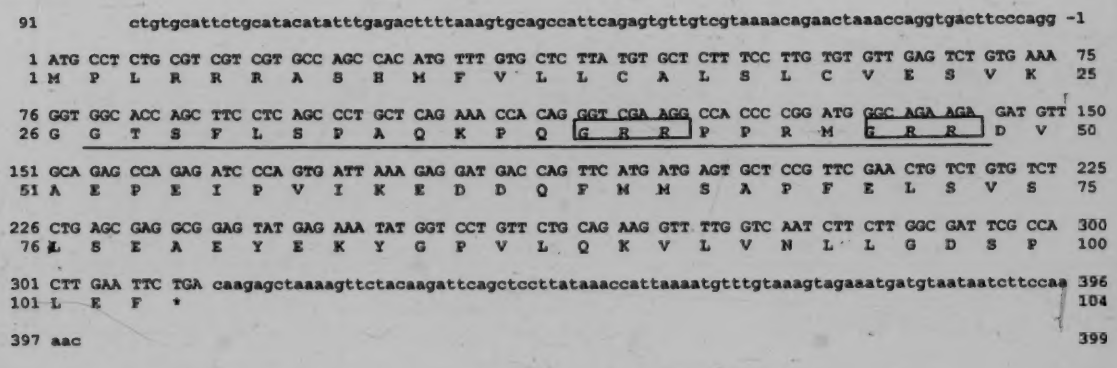
91 ctgtgcattctgcatacatatttgagacttttaaagtgcagccattcagagtgttgtcgtaaaacagaactaaaccaggtgacttcccagg -1
1 ATG CCT CTG CGT CGT CGT GCC AGC CAC ATG TTT GTG CTC TTA TGT GCT CTT TCC TTG TGT GTT GAG TCT GTG AAA 75
1 M P L R R R A S H M F V L L C A L S L C V E S V K 25
76 GGT GGC ACC AGC TTC CTC AGC CCT GCT CAG AAA CCA CAG GGT CGA AGG CCA CCC CGG ATG GGC AGA AGA GAT GTT 150
26 G G T S F L S P A Q K P Q G R R P P R M G R R D V 50
151 GCA GAG CCA GAG ATC CCA GTG ATT AAA GAG GAT GAC CAG TTC ATG ATG AGT GCT CCG TTC GAA CTG TCT GTG TCT 225
51 A E P E I P V I K E D D Q F M M S A P F E L S V S 75
226 CTG AGC GAG GCG GAG TAT GAG AAA TAT GGT CCT GTT CTG CAG AAG GTT TTG GTC AAT CTT CTT GGC GAT TCG CCA 300
76 L S E A E Y E K Y G P V L Q K V L V N L L G D S P 100
301 CTT GAA TTC TGA caagagctaaaagttctacaagattcagctccttataaaccattaaaatgtttgtaaagtagaaatgatgtaataatcttccaa 396
101 L E F * 104
397 aac 399

Figure 2.1. Nucleotide sequence of the goldfish preproghrelin cDNA. The mature peptide region is underlined. The two cleavage sites and amidation signals are boxed.

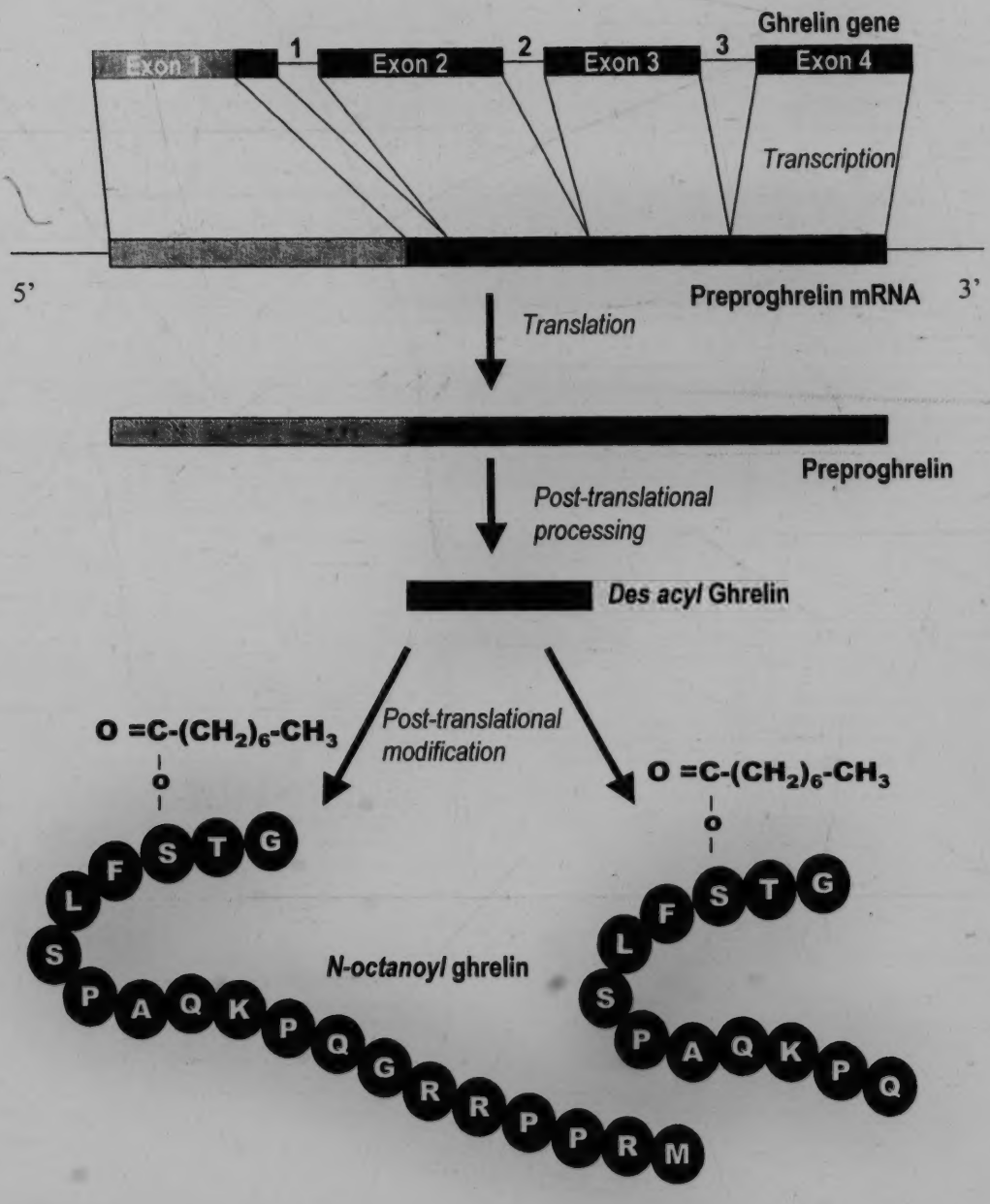

Figure 2.2. Proposed model of the formation of n-octanoylated ghrelin mature peptides from the ghrelin gene. The exons are represented by boxes and introns are represented by thin lines.

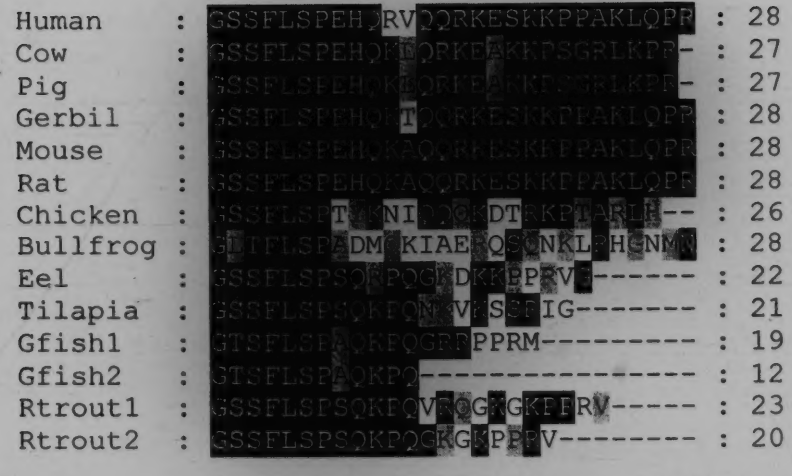
Human : GSSFLSPEHQRVQQRKESKKPPAKLQPR : 28
Cow : GSSFLSPEHQRLQQRKEAKKPSGRLKPR- : 27
Pig : GSSFLSPEHQKLQQRTEAKKPSGRLKPR- : 27
Gerbil : GSSFLSPEHQETQQRKESKKPPAKLQPR : 28
Mouse : GSSFLSPEHQKAQQRKESKKPPAKLQPR : 28
Rat : GSSFLSPEHQRAQQRKESKKPPAKLQPR : 28
Chicken : GSSFLSPTVSNIQQQKDTKKPTARLR-- : 26
Bullfrog : GLTFLSPADMQKIAERQSQNKLRHGNMN : 28
Eel : GSSFLSPSQRPQGKDKKPPRVE------ : 22
Tilapia : GSSFLSPSQKPQNEVESSPIG------- : 21
Gfish1 : GTSFLSPAQKPQGRPPPRM--------- : 19
Gfish2 : GTSFLSPAQKPQ---------------- : 12
Rtrout1 : GSSFLSPSQKPQVRQGKGKPPRV----- : 23
Rtrout2 : GSSFLSPSQKPQGKGKPPPV-------- : 20

Figure 2.3. Comparison of the mature peptide sequences of ghrelin from various other species. 'Gfish' stands for goldfish and 'Rtrout' stands for rainbow trout. Sequences used for the alignment were obtained from the GenBank.

Figure 2.4. (A) RT-PCR analysis of the tissue distribution of ghrelin mRNA in the brain and peripheral tissues of goldfish. The different lanes represent the following: 1 and 20, molecular weight markers; 2, olfactory bulbs and tract; 3, telencephalon; 4, hypothalamus; 5, midbrain; 6, posterior brain; 7, pituitary; 8, gut; 9, liver; 10, spleen; 11, muscle; 12, gill; 13, heart; 14, kidney; 15, eye; 16, testis; 17, ovary; 18, negative control (primers and PCR reaction mix only, no template); 19, positive control (template used was the diluted plasmid miniprep of goldfish ghrelin cDNA). **(B)** Southern blot on the same gel using a ^{32}P-labeled probe for full length ghrelin **(C)** β-actin PCR as an internal control for all cDNAs used for the distribution PCR.

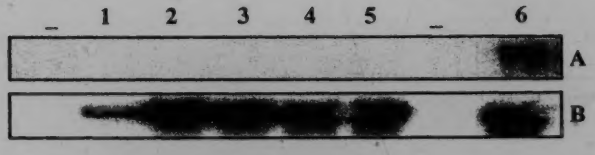

Figure 2.5. (A) Northern blot showing the distribution of ghrelin mRNA in the brain and gut of goldfish. The different lanes represent the following: 1, olfactory bulbs and tracts; 2, telencephalon; 3, hypothalamus; 4, midbrain; 5, hindbrain; and 6, gut. **(B)** The membrane was stripped and reprobed with the β - actin probe.

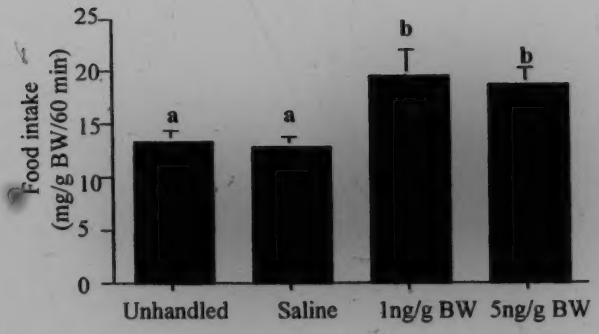

Figure 2.6. Effects of ICV injection of goldfish ghrelin (1-19) on food intake. Fish were unhandled (n = 12), saline injected (n = 8) or injected with 1ng/g BW (n = 8) or 5 ng/g BW (n = 8) goldfish ghrelin (1-19). Data is presented as mean±SEM. Values with different letters are significantly different ($p<0.05$).

CHAPTER 3

OREXIGENIC ACTIONS OF GHRELIN IN GOLDFISH: FEEDING INDUCED CHANGES IN BRAIN AND GUT mRNA EXPRESSION AND SERUM LEVELS, AND RESPONSES TO CENTRAL AND PERIPHERAL INJECTIONS

3.1. Introduction

Ghrelin is a recently identified endogenous ligand of the growth hormone secretagogue receptor (GH-R) (Kojima et al., 1999). Ghrelin has a unique, *n-octanoyl* modification in the third residue, serine, of the mature peptide (Kojima et al., 1999). This modification is essential for its biological activity (Hosoda et al., 2000). The structures of the ghrelin gene and the ghrelin peptide has been identified from several species (Cummings and Shannon, 2003). Preproghrelin mRNA expression has been detected in the stomach and the hypothalamus of mammals (Kojima et al., 1999, Gnanapavan et al., 2002). Ghrelin immunoreactivity has been found in the stomach (Kojima et al., 1999, Gnanapavan et al., 2002) and the brain of mammals (Kojima et al., 1999, Cowley et al., 2003). Ghrelin neurons are in contact with the cell bodies and dendrites of neuropeptide Y (NPY), agouti related protein (AgRP) and proopiomelanocortin (POMC) neurons in the hypothalamic arcuate nuclei of rats (Cowley et al., 2003). The stomach, however seems to be the primary site of production of ghrelin in mammals (Sakata et al., 2002). Ghrelin binding sites

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are present in the hypothalamus of rat brain (Cowley et al., 2003).

Intracerebroventricular (ICV) injections of ghrelin stimulate food intake in rats (Nakazato et al, 2001, Wren et al., 2001) and mice (Tschop et al., 2000). Intraperitoneal (IP) administration of ghrelin induce hunger, feeding and adiposity in rats (Wren et al., 2001). Furthermore, NPY and AgRP have been shown to mediate the central actions of ghrelin on energy balance in mammals (Cowley et al., 2003, Tschop et al., 2000). A preprandial increase and a postprandial decrease in plasma ghrelin levels has been demonstrated in humans (Cummings et al., 2001). These results suggest that ghrelin acts as a "meal initiator" in humans (Cummings et al., 2001).

Recently, the structure of the ghrelin gene in goldfish, a teleost fish that lacks a well demarcated stomach, has been identified (Chapter 2). There are two putative cleavage sites and amidation signals in the goldfish ghrelin gene suggesting the presence of two ghrelin peptides. One of the putative peptides, the short ghrelin is 12 amino acid in length ($gGRL_{[1-12]}$) and the second, long ghrelin is 19 amino acid in length ($gGRL_{[1-19]}$). Although the ghrelin peptides have not been purified from the goldfish, previous studies suggest that two peptides form from a prepropeptide mRNA with two putative cleavage sites and amidation signals. For example, two peptides, orexin-A and orexin-B, originate from prepro-orexin mRNA that has two cleavage sites and two amidation signals (Sakurai et al., 1999). The N-terminal seven amino acids of ghrelin (GSSFLSP) are identical in mammals (Kojima et al., 1999, Cummings et al., 2003), chicken (Kaiya et al., 2002), eel (Kaiya et al., 2003)

and tilapia (Parhar et al., 2003); while in bullfrog (GLTFLSP) (Kaiya et al., 2001) and goldfish (GTSFLSP) (Chapter 2) some amino acids are substituted. Goldfish ghrelin (12 or 19 amino acids) and tilapia ghrelin (20 amino acids) are shorter than the mammalian (28 amino acids), avian (26 amino acids), amphibian (28 amino acids) and eel (27 or 28 amino acids) ghrelin. RT-PCR followed by Southern blot analysis detected preproghrelin mRNA expression in the gut and also in brain regions (telencephalon and hypothalamus) involved in the regulation of food intake in goldfish (Chapter 2). ICV injection of 1 and 5 ng/g bodyweight (BW) of *n-octanoylated* $gGRL_{[1-19]}$ stimulates food intake in goldfish (Chapter 2). The biological activity of $gGRL_{[1-12]}$ is currently unknown.

The objectives of the studies discussed in this chapter are to identify, (i) the preprandial-, postprandial- and starvation-induced changes in preproghrelin mRNA expression in the hypothalamus and gut, (ii) the preprandial, postprandial and fasting induced changes in serum ghrelin levels, and (iii) the effects of [a] ICV administration of the putative native peptide, *n-octanoylated* $gGRL_{[1-12]}$ and synthetic human ghrelin and [b] IP administration of putative native peptides, *n-octanoylated* $gGRL_{[1-12]}$ and $gGRL_{[1-19]}$ and synthetic human ghrelin on food intake in goldfish.

3.2. Experiment Design, Materials and Methods

Animals

Male and female goldfish (*Carassius auratus*) of the common or comet varieties and approximately 40-50 g body weight, were purchased from the Mount

Parnell Fisheries (Mercersburg, PA, USA) and maintained in 65 l aquaria at 20 C under a simulated photoperiod of 16 h light and 8 h dark. Fish were fed once daily at a scheduled feeding time with pellet fish diet (Corey Aquafeeds, Fredericton, New Brunswick, Canada). Fish were acclimatized to the above described conditions for a minimum of four weeks. Fish were anesthetized by immersing in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) solution before decapitation and dissection of the brain and gut. All protocols adhered to the policies of the Canadian Council of Animal Care and were approved by the Faculty of Science Animal Care Committee, University of Alberta.

Ghrelin

Octanoylated $gGRL_{[1-12]}$ and $gGRL_{[1-19]}$ (12) were synthesized using the solid phase (9 fluorenylmethoxycarbonyl) strategy at the laboratory of Dr. Jean E Rivier, Clayton Foundation Laboratories for Peptide Biology, The Salk Institute of Biological Sciences (San Diego, California, USA). Since the ghrelin that is predominantly present in the goldfish is not known, both putative peptides ($gGRL_{[1-12]}$ and $gGRL_{[1-19]}$) for the *in vivo* studies. Human ghrelin was purchased from the Phoenix pharmaceuticals (California, USA). Peptides were freshly dissolved in fish physiological saline (Burnstock, 1958) and aliquoted for each injection dosage. Tubes containing the test solutions were blind labeled so that the identity of the test solution was unknown to the researcher during the injections.

Sampling plan for tissue collection

(a) Preprandial and postprandial experiment

Seven groups of fish (tissues from 5 fish pooled together to make each sample, n = 5), all at a similar stage of sexual development (sexually recrudescent stage), were kept in 65l glass aquaria under the same photoperiod and temperature conditions. Fish were fed daily at a scheduled time (1500 h) and allowed to acclimatize to the tank conditions and feeding time for a minimum of eight weeks. Previously, we used the total RNA extracted from the beginning of the gastrointestinal tract including the "J" region (Romer, 1971) to clone the preproghrelin cDNA (Chapter 2). In this study also, we used the above described region of the gastrointestinal tract (referred as the "gut") for total RNA extraction. On the first sampling day, fish from one group each were euthanized at 3h (1200 h) and 1h (1400 h) before the scheduled feeding time and at the scheduled feeding time (1500 h), and tissue samples (telencephalon, hypothalamus and gut) collected. Two groups of fish remained unfed at the regular feeding time and were sampled at 1 h (1600 h) and 3h (1800 h) after the scheduled feeding time and tissues collected. On the second sampling day, two other groups of fish were fed at the scheduled feeding time (1500 h) and sampled at 1h (1600 h) and 3h (1800 h) after feeding. Tissues were stored at $^{-}$80 C until RNA extraction. In previous studies in our laboratory, preprandial and postprandial changes in NPY mRNA expression were detected in goldfish 3 h prior to and 3 h after the scheduled feeding time (Narnaware et al., 2000).

(b) *Starvation experiment*

Four groups of fish (n = 8) were acclimatized as described in the pervious section. One group was sampled (for collecting the telencephalon, hypothalamus and gut) on day 1, after 24 h starvation (same as the 0 h group in the feeding experiment). The other three groups remained unfed and tissues were collected on days 3, 5 and 7 at 1700 h. Tissues were stored at ¯80 C until RNA extraction.

Sampling plan for serum collection

For the preprandial/postprandial experiment, seven groups of fish (n = 7) were acclimatized as described in the previous section and blood samples were collected from each fish by caudal puncture according to the schedule described earlier. For the starvation experiment, five groups (four groups as described in the previous section plus an additional group for fed controls) of fish (n = 7) were acclimatized as described previously. On day 1 of the experiment, one group of fish was sampled at the regular feeding time (after 24 h starvation, same as the 0 h group in the feeding experiment). Another group was fed at the regular feeding time and sampled three h after feeding. This group served as the control. The remaining three groups were kept unfed and one group was sampled each on days 3, 5 and 7 at the regular feeding time. Approximately 0.5 ml of blood from each fish was collected by caudal puncture using a 1 ml syringe (Beckton Dickson Co., New Jersey, USA). Blood samples were stored in 1.6 ml flat top microfuge tubes (Fisher Scientific) at 4 C for 1 h to allow clotting, and then spun for 5 min at 8000 rpm in a microfuge. Collected serum was stored at ¯20 C until the ghrelin radioimmunoassay (RIA).

Slot blot analysis of ghrelin mRNA expression

(a) Total RNA extraction

Total RNA was extracted from the telencephalon, hypothalamus and gut of goldfish using Trizol reagent (Invitrogen Life Technologies, Ontario, Canada). The integrity of the RNA was determined using a spectrophotometer at 260/280 nm. The RNA samples were kept at ¯80 C until the slot blot analysis was carried out.

(b) Standardization of the slot blot analysis

Northern blot analysis did not detect preproghrelin mRNA expression in the brain of any of the species studied (Kojima et al., 1999, Chapter 2). The suitability of slot blot analysis for the quantification of preproghrelin mRNA was tested. In order to use slot blot for the quantification of the preproghrelin mRNA, it is essential to (i) determine the sensitivity of slot blot analysis to quantify the preproghrelin mRNA in the brain regions (telencephalon, hypothalamus, mid brain and hindbrain) and gut and (ii) determine the appropriate amount of total RNA required from each tissue. In order to achieve this, a serial dilution of total RNA from telencephalon, hypothalamus, midbrain, hindbrain and gut was blotted into a Hybond Nylon membrane (Amersham Life Sciences UK Ltd, Buckinghamshire) using the Bio-Dot SF blotting apparatus (Bio-Rad Laboratories, Hercules, California, USA). Total RNA used for the standardization of slot blot was pooled from 10 fish kept under the experimental conditions. A specific cDNA probe covering the full length (490 bp) of goldfish preproghrelin (Chapter 2) was amplified as described previously (Chapter 2). The probes were labeled with [α-^{32}P]dCTP using a Rediprime II – Quickprime

kit (Qiagen Inc., Ontario, Canada) following the manufacturer's protocol. The labeled probe was then purified using a QIAquick nucleotide removal kit (Qiagen) following the manufacturer's instructions. 10^6 cpm/ml of probe were used for hybridization. Hybridization was performed using methods described by Canosa et al. (2002). After hybridization overnight at 65 C, the membranes were washed twice with washing solution (0.04 M $NaHPO_4$, 1mM EDTA, and 1% SDS) at 65 C. The membranes were then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 10 days and the screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). As an internal control, the membranes were stripped and reprobed with an [α-^{32}P]dCTP labeled goldfish β-actin (GenBank accession number AF079831) probe. Standardization of the slot blot analysis was conducted thrice for each tissue.

(c) *Slot blot analysis of the experimental samples*

Based on the standardization values obtained for each tissue, an appropriate quantity (5 μg) of total RNA from the telencephalon, hypothalamus and gut experimental samples were used. In order to reduce variability, the control and experimental samples from each experiment were blotted to the same nylon membrane. Slot blot analyses were conducted as described in the previous section. Slot blot analysis was conducted twice for each experiment.

Ghrelin RIA

Serum ghrelin levels were detected using the ghrelin (active) RIA kit (Catalog number GHRA-88HK, Linco Research, Inc., Missouri, USA). This assay has a specific, guinea pig antibody that detects only the active (*n-octanoylated*) form of ghrelin. This assay does not detect the inactive (*des-octanoyl*) ghrelin, leptin, glucagon-like-peptide, insulin and motilin-related-peptide. The upper and lower limits of sensitivity of the assay are 2000 pg/ml and 10 pg/ml, respectively. The performance parameters for the assay are $ED_{80} = 21 \pm 5$ pg/ml, $ED_{50} = 105 \pm 5$ pg/ml and $ED_{20} = 565 \pm 20$ pg/ml. The intraassay and interassay coefficients of variance are 6.7% and 9.6%, respectively. The RIA was conducted according to the manufacturer's protocol, except that no acid was added to the serum samples and duplicate 50 µl samples of serum were used. To validate the RIA, serum from several fishes were pooled, serially diluted (1,1/2,1/4, 1/8, 1/16, 1/32) and assayed using the kit following the manufacturer's protocol. Additionally, goldfish serum alone or with added synthetic *n-octanoylated* $gGRL_{[1-19]}$ (1570 pg) were assayed to determine the efficiency of the assay to detect the *n-octanoylated* goldfish ghrelin. Quality controls provided by the manufacturer were included in the assay. All samples were assayed in duplicate. All samples from a single experiment were included in one assay to reduce variability. Preliminary analysis did not find any difference in serum ghrelin levels in male and female goldfish undergoing recrudescent stage of sexual development.

ICV injections

ICV injections were conducted as described earlier (Chapter 2). Briefly, male or female fish (n = 8; size selected for later injections), undergoing late gonadal recrudescent stage were anesthetized as described previously, placed on a sponge holder and a three sided flap was cut in the skull using a circular saw attached to a dentist drill. The flap was then folded to one side to expose the brain. Fish were then placed on a stereotaxic apparatus. Using the coordinates (+1.0, M, D 1.2) described in the goldfish brain atlas (Peter and Gill, 1975), the needle of a 5 µl Hamilton syringe (Hamilton Co., Nevada, USA) was placed in the preoptic region of the brain third ventricle, and 2 µl of fish physiological saline, or the test solution containing a specific concentration of $gGRL_{[1-12]}$ or human ghrelin was injected. The needle was then withdrawn, the cranial cavity filled with fish physiological saline and the flap returned to its original location and sutured. Fish were then immediately placed back in their aquarium and allowed to recover. Normal recovery time was within 5 min. After 15 min, the food pellets were administered (4% of BW per fish) and food intake of each fish was measured for 1 h. Preliminary studies in our laboratory have found that injection of India ink into the brain ventricle using the coordinates mentioned above resulted in the distribution of the dye throughout the entire brain ventricular system within 5 min (Volkoff, H., personal communication). Cumulative food intake by male and female goldfish undergoing gonadal recrudescence stage was similar (Himick and Peter, 1995).

IP injections

Fish undergoing the gonadal recrudescence stage were anesthetized as described previously and 100 µl of fish physiological saline or 100 µl of fish physiological saline containing the specified concentration of $gGRL_{[1-12]}$, $gGRL_{[1-19]}$ or human ghrelin was injected into the peritoneal cavity using a 26-gauge needle (Beckton Dickson Co.) attached to a 250 µl Hamilton syringe (Hamilton Co.). Fish were then immediately placed back in their aquarium and allowed to recover. Normally the fish recovered within 2 min. Food pellets (4% BW per fish) were administered 15 min after the reentry of the fish in to the aquarium and food intake of each fish was measured for 1 h.

Quantification of food intake

Experiments were conducted at the regular feeding time. To conduct the tests, two fish were anesthetized and were blind injected with the fish physiological saline or the test solution. Quantification of food intake was made using a previously described method (Chapter 2). Food intake was converted to milligrams of food consumed per gram wet bodyweight of the fish for 60 min based on the average weight of the pellet given. Stressed fish or fish in which there were problems with the suturing after the ICV injections were removed from the tanks and euthanized.

Statistical analysis

Statistical analyses were conducted using ANOVA followed by Tukey's test. Data are expressed as mean ± SEM. The preproghrelin mRNA levels were expressed as a ratio of the preproghrelin mRNA to β actin mRNA and then

normalized as a percentage of the expression levels at $^{-}$3h (for the preprandial/postprandial experiment) or the expression levels in day 1 (for the starvation experiment). Pooled data from two different experiments were shown for the starvation induced changes in preproghrelin mRNA expression in the hypothalamus and gut. Postprandial ($^{+}$3h and $^{+}$1h) preproghrelin mRNA expression and serum ghrelin levels in fed fish and the unfed fish were compared using Independent t-test. The slopes of the serial dilution of goldfish serum and the synthetic standards were compared by ANOVA using a computer program (Stefano et al., 1997). Significance was considered at $P<0.05$.

3.3. Results

Quantification of preproghrelin mRNA expression using slot blot

Quantifiable signals of preproghrelin mRNA were obtained from the telencephalon (data not shown), hypothalamus and gut of goldfish. No signals were obtained from the midbrain or hind brain of goldfish (data not shown). Figure 3.1.A (hypothalamus) and Figure 3.1.B (gut) show the linear relationships between the serial dilution of total RNA and the preproghrelin signal. The results indicate that 5 μg total RNA is an appropriate quantity for slot blot analysis of experimental samples from the telencephalon, hypothalamus and gut.

Preprandial and postprandial changes in preproghrelin mRNA expression

In the groups that were unfed at the regular feeding time, the preprandial and postprandial preproghrelin mRNA expression in the hypothalamus (Figure 3.2. A) were unchanged. In the groups that were fed at the regular feeding time, a

significant postprandial decrease in preproghrelin mRNA expression occurred in the hypothalamus (Figure 3.2.A). A significant decrease in preproghrelin mRNA expression was detected in the gut of unfed fish at 3h after the scheduled feeding time, compared to the expression levels at ¯1h and 0h (Figure 3.2.B). In the fed fish, a similar postprandial decrease in preproghrelin mRNA was detected in the gut (Figure 3.2.B). However, the postprandial preproghrelin mRNA expression in the gut at 3 h after meal intake in the fed group was significantly lower than the expression levels in the unfed group at the same time (denoted by an asterisk in Figure 3.2.B). No significant changes were detected in the preproghrelin mRNA expression in the telencephalon (data not shown).

Starvation induced changes in preproghrelin mRNA expression

A significant increase in preproghrelin mRNA expression in the hypothalamus was found on day 7 relative to the preproghrelin mRNA expression in day 1 (Figure 3.3.A). Similarly, the preproghrelin mRNA expression in the gut on day 7 was higher compared to its expression on day 1 (Figure 3.3.B). No significant changes were detected in the preproghrelin mRNA expression in the hypothalamus (Figure 3.A) and gut (Figure 3.3.B) on days 3 and 5. During the 7 day starvation, no significant changes in preproghrelin mRNA expression were detected in the telencephalon (data not shown).

Validation of the ghrelin RIA

Validation of the ghrelin (active) RIA using a serial dilution of goldfish serum showed parallelism with respect to the standards provided with the assay kit

(Figure 3.4). No significant differences were found between the slopes. Synthetic ghrelin added to goldfish serum was accurately detected (goldfish serum alone = 278 ± 31.6 pg/ml and goldfish serum plus 1570 pg of $gGRL_{[1-19]}$ = 1823.1 ± 70.9 pg/ml). The quality controls were within the ranges provided by the manufacturer.

Preprandial and postprandial changes in serum ghrelin levels

A significant postprandial decrease in serum ghrelin levels was found (Figure 3.5.A). In the fish that were unfed at the scheduled feeding time, the serum ghrelin levels remained unchanged (Figure 3.5.A). The postprandial serum ghrelin levels at 1h and 3h after the meal were significantly lower than the serum ghrelin levels in the unfed fish at the same sample time (denoted by asterisks in Figure 3.5.A).

Starvation induced changes in serum ghrelin levels

A significant increase in serum ghrelin levels was observed in days 3 and 5 of fasting, but the levels decreased to control levels at day 7 of fasting (Figure 3.5.B).

Effect of ICV injection of $gGRL_{[1-12]}$ and human ghrelin on food intake

ICV administration of 10 ng/g BW of $gGRL_{[1-12]}$ stimulated food intake (Figure 3.6.A) compared to the unhandled controls. ICV administration of 1 and 10ng/g BW (Figure 3.6.A) of human ghrelin stimulated food intake (Figure 3.6.B) compared to the unhandled and saline treated controls.

Effect of IP injections of $gGRL_{[1-12]}$, $gGRL_{[1-19]}$ and human ghrelin on food intake

IP administration of 10 ng/g BW of $gGRL_{[1-12]}$ (Figure 3.7. A), 100 ng/g BW of $gGRL_{[1-19]}$ (Figure 7. B) and 10 and 100 ng of human ghrelin (Figure 3.7.C) stimulated food intake compared to the unhandled and saline treated controls.

3.4. Discussion

The results presented in this chapter indicate that slot blot analysis detects preproghrelin mRNA expression in the telencephalon and hypothalamus of goldfish. Slot blot analyses has been successfully used for identifying mRNAs encoding regulatory peptides and their receptors in mammals (Bagnasco et al., 2002) and in the goldfish (Canosa et al., 2002, Cerda-Reverter and Peter, 2003a, Volkoff and Peter, 2002). Although in a slot blot the total RNA is not separated on a gel and each slot contains mRNAs encoding many messages, the empty slots were devoid of any signals. Additionally, no signals were detected in the midbrain and hindbrain of goldfish, suggesting that the signal detected in the telencephalon and hypothalamus was of preproghrelin mRNA. Preliminary in situ hybridization studies using a radioactive ghrelin probe and prolonged exposure (45 days) detected a weak signal in the telencephalon and hypothalamus of goldfish brain. However, due to the high background, it was difficult to photograph and quantify the signal.

These results demonstrate that preproghrelin mRNA expression occurs in the hypothalamus and gut and that the serum ghrelin levels change during the scheduled feeding time in goldfish. Starvation induced an increase in preproghrelin mRNA expression in the hypothalamus and gut on day 7, while significant increases in serum ghrelin levels were found on days 3 and 5. To the best of my knowledge, this is the first report on the preprandial, postprandial and starvation induced changes in preproghrelin mRNA expression in the brain of any species. Also, this is the first report on the preprandial and postprandial serum ghrelin profile in a non-mammalian

vertebrate. mRNAs encoding many other orexigenic and anorexigenic factors, including the NPY (Narnaware et al., 2000), AgRP (Cerda-Reverter and Peter, 2003a), galanin (Unniappan et al., 2003) and the cocaine and amphetamine regulated transcript (CART) (Volkoff and Peter, 2002) are expressed in the hypothalamus of goldfish. The melanocortin receptor (MC-4R) mRNA is also expressed in the hypothalamus of goldfish (Cerda-Reverter et al., 2003b). A preprandial increase and a postprandial decrease in NPY mRNA expression in the hypothalamus of goldfish has been demonstrated (Narnaware et al., 2000). Fasting increases NPY (Narnaware et al., 2000) and AgRP (Cerda-Reverter and Peter, 2003a) mRNA expression in the hypothalamus of goldfish. Changes in the expression of ghrelin in these brain areas suggest that ghrelin acts centrally to regulate food intake in goldfish.

Seven day fasting results in approximately 10% reduction in the bodyweight of goldfish (Cerda-reverter JM, personal communication). The fish were less active by the seventh day, but returned to normalcy after refeeding (Cerda-reverter JM, personal communication). It is notable that the preproghrelin gene expression in the hypothalamus and gut of goldfish on days 3 and 5 of starvation is not significantly higher than the control. On day 7, there is an increase in the preproghrelin mRNA expression in the hypothalamus and the gut. On the contrary, the serum ghrelin levels are significantly elevated (approximately 300% higher compared to the fed controls) on days 3 and 5 of starvation and decrease to control levels on day 7. The reasons behind these apparent disjunct changes in serum ghrelin levels and ghrelin expression are currently not known. Fasting increases and refeeding decreases

plasma ghrelin levels in normal and Zucker fatty rats (Ariyasu et al., 2002). 48 h fasting increases both preproghrelin mRNA expression in the gastric fundus and plasma ghrelin levels in rats (Ariyasu et al., 2002). However, a recent report did not find any changes in the preproghrelin mRNA expression in the stomach of tilapia, *Oreochromis niloticus* after 7 days of fasting (Parhar et al., 2003). In burbot *(Lota lota)*, fasting reduced plasma ghrelin levels at 2 C, while plasma ghrelin levels remained unchanged in burbot kept at 10 C (Nieminen et al., 2003). Some physiological functions of ghrelin are species specific (eg: ghrelin stimulates prolactin release in Japanese eel [Kaiya et al., 2003], while it has no effect on prolactin release in the rainbow trout [Kaiya et al., 2003]). Likewise, the difference in the results of our starvation experiments in goldfish and the results of a similar studies conducted in tilapia [Parhar et al., 2003] and burbot (Nieminen et al., 2003) might be due to species-specific physiological differences.

Central injections of $gGRL_{[1-12]}$ and human ghrelin and peripheral injections of $gGRL_{[1-19]}$, $gGRL_{[1-12]}$ and human ghrelin stimulate food intake in goldfish. Our results show that both $gGRL_{[1-19]}$ and $gGRL_{[1-12]}$ are biologically active in goldfish. However, the magnitude of increase in food intake after central injections of $gGRL_{[1-12]}$ was less compared to that of the $gGRL_{[1-19]}$ (Chapter 2) and human ghrelin. Central administration of ghrelin stimulates food intake in rats (Nakazato et al., 2001, Wren et al., 2001) and mice (Tschop et al., 2000) and induced AgRP (Kamegai et al., 2000, Seoane et al., 2003) and NPY (Kamegai et al., 2000, Seoane et al., 2003, Kamegai et al., 2000) mRNA expression in rats. It has been shown that ghrelin acts

directly on the NPY neurons in the rat arcuate nuclei (Kohno et al., 2003). Either the blockade of NPY (Nakazato et al., 2001) or AgRP receptors (Wren et al., 2001) or the immunoneutralization of NPY or AgRP (Nakazato et al., 2001) resulted in the attenuation of ghrelin's orexigenic action. IP administration of ghrelin stimulated *c-fos* expression only in the NPY neurons present in the mouse hypothalamic arcuate nuclei, suggesting that NPY neurons in the hypothalamus are the primary target of peripherally administered ghrelin (Wang et al., 2002). Recently, the role of orexin in mediating the orexigenic actions of ghrelin has been demonstrated (Toshinai et al., 2003). Stimulatory effects of ghrelin on food intake were reduced in orexin knockout mice (Toshinai et al., 2003). In goldfish, the structure and pattern of actions of appetite regulatory peptides are highly conserved (Lin et al., 2000). ICV administration of NPY (Narnaware et al., 2000), MC-4R antagonists (Cerda-Reverter et al., 2003b) and orexins (Volkoff et al., 2003) stimulate food intake in goldfish. It is possible that in goldfish, ghrelin may also interact with the NPY, AgRP, orexins and other orexigens and anorexigens to regulate food intake. These questions are currently being studied in our laboratory.

In summary, the preprandial, postprandial and starvation induced changes in preproghrelin mRNA expression in the goldfish brain are reported in this chapter. ICV and IP administration of $gGRL_{[1-12]}$ and human ghrelin and IP administration of $gGRL_{[1-19]}$ stimulates food intake in goldfish suggesting that ghrelin may act via central and peripheral mechanisms controlling appetite in goldfish. These results also suggest that $gGRL_{[1-12]}$, the putative short peptide formed by alternative

processing of the preproghrelin peptide is biologically active. Collectively, these findings suggest that ghrelin is an orexigen in goldfish. It also suggests that, the synthesis, secretion and actions of ghrelin on food intake are highly conserved during vertebrate evolution.

3.5. REFERENCES

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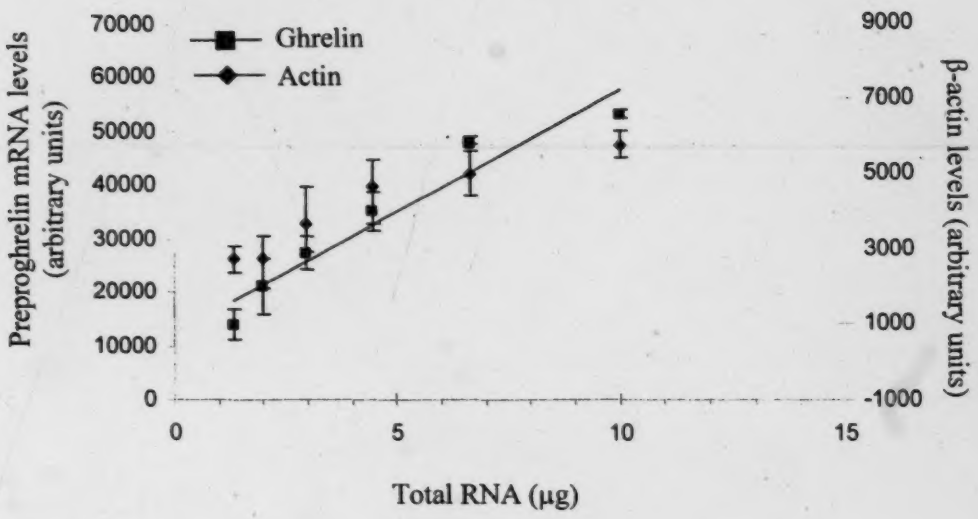
A Hypothalamus



B Gut

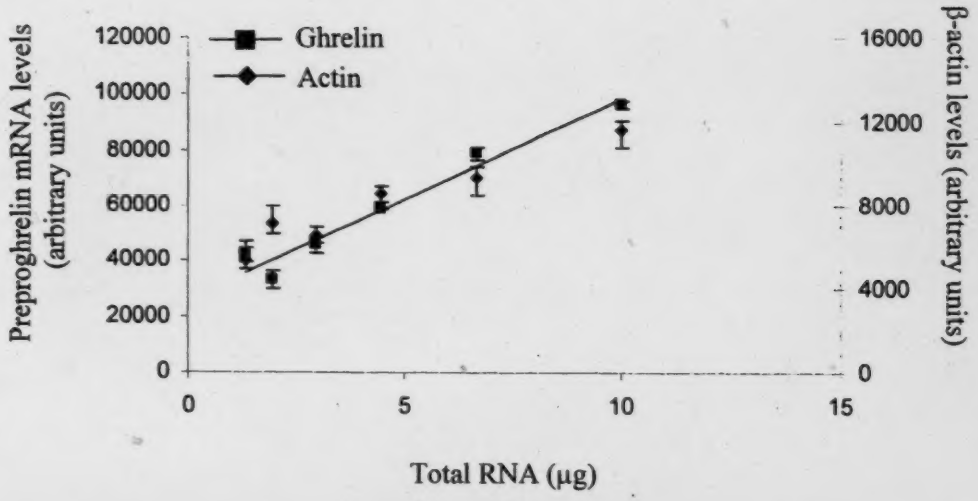


Figure 3.1. Linear range of hybridization signal (optical density). Serial dilutions of total RNA from the hypothalamus (A) or gut (B) were transferred to Nylon membranes using the slot blot apparatus and hybridized with goldfish preproghrelin probe, and then stripped and reprobed with goldfish β actin probe as an internal control.

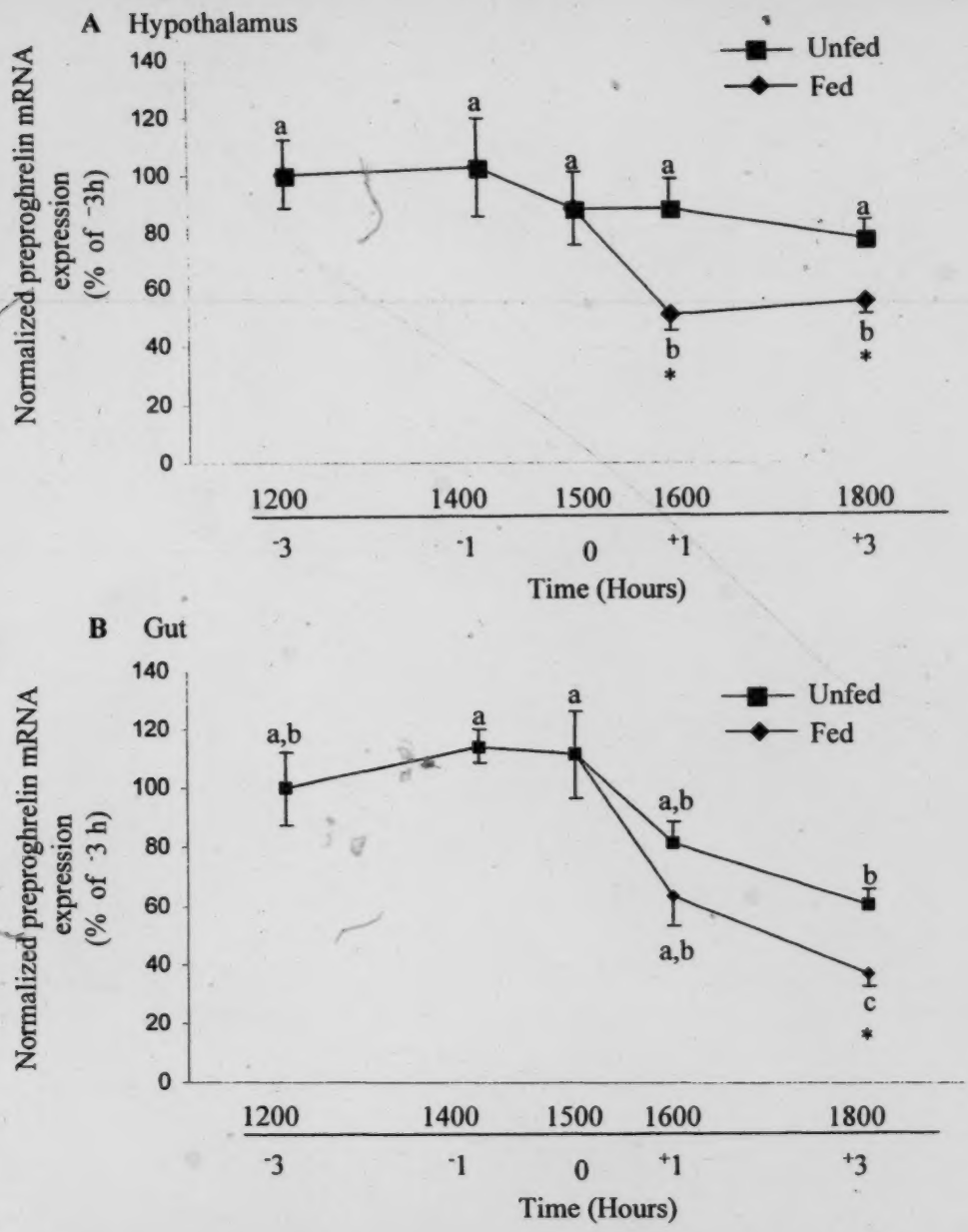


Figure 3.2. Preprandial and postprandial changes in the expression of preproghrelin mRNA in the hypothalamus (A) and gut (B). Figures show the pre- and postprandial mRNA expression data as the normalized percentage of the preproghrelin mRNA expression in the -3 group (3 h before the scheduled feeding time). Data is presented as mean±SEM. n = 5. Same letters denote values that are not significantly different. Asterisks represent significant differences between groups of the same time point.

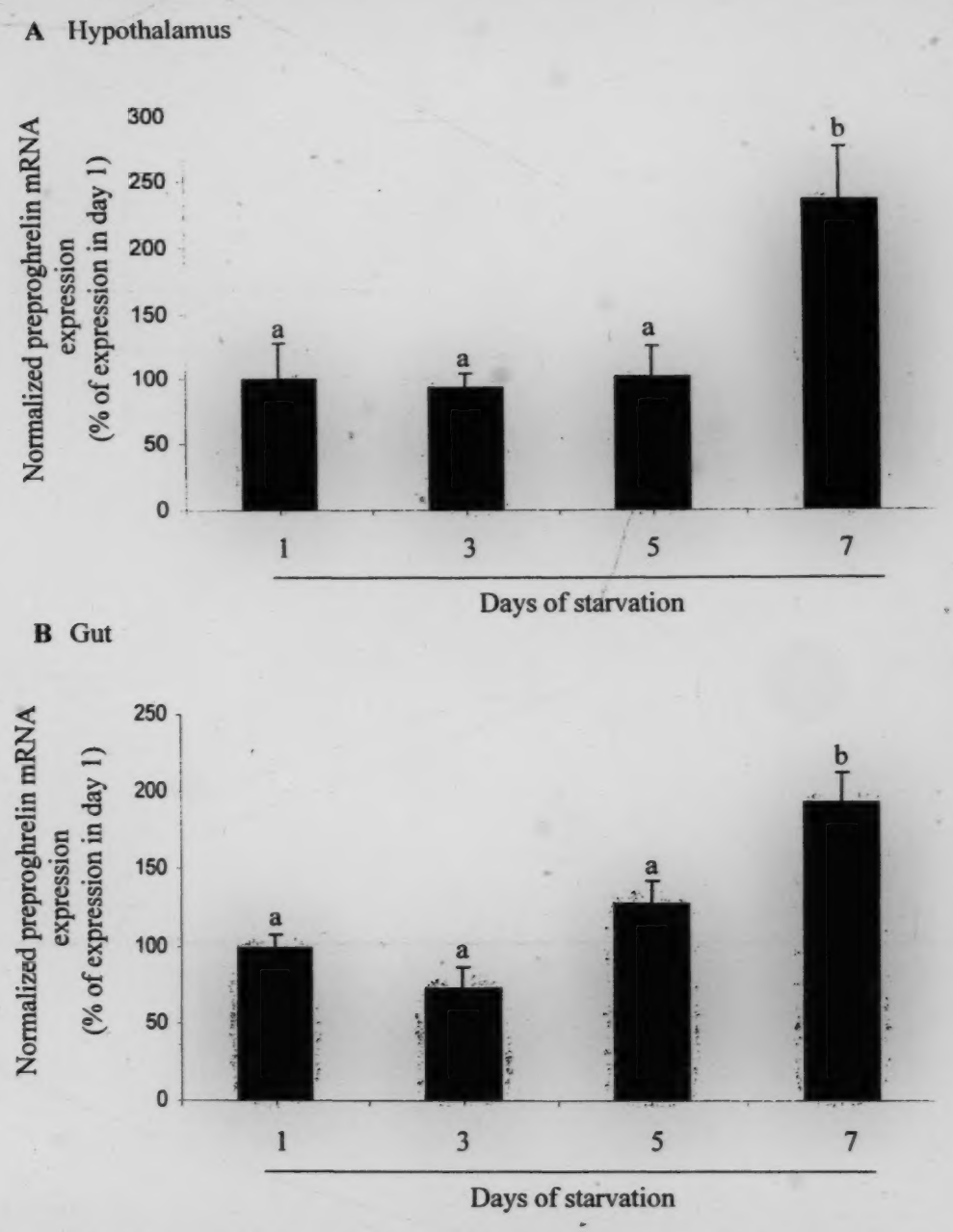


Figure 3.3. Starvation induced changes in the preproghrelin mRNA expression in the hypothalamus (A) and gut (B) of goldfish. Pooled data from two experiments are shown. Data shown are normalized as percentage of the preproghrelin mRNA expression in fish from day 1 (24h fasted fish). Data is presented as mean±SEM. n = 8. Same letters denote values that are not statistically different.

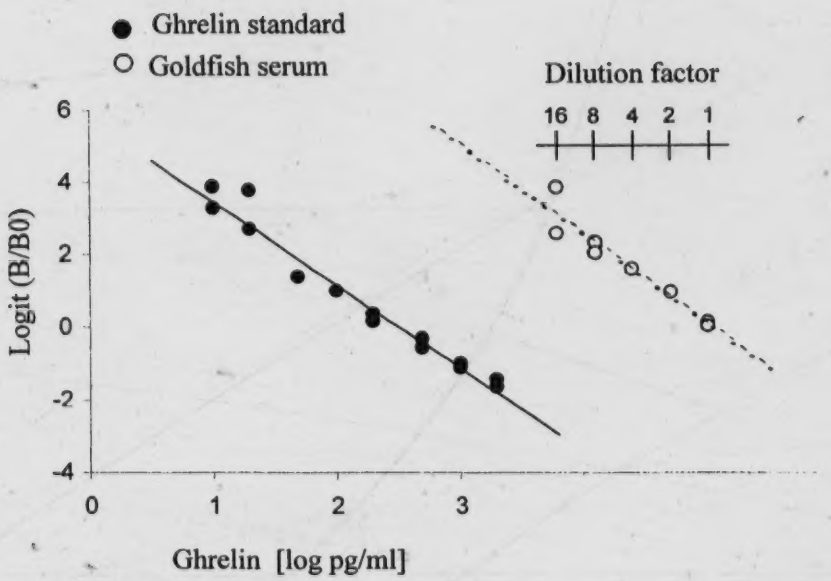


Figure 3.4. Validation of the ghrelin (active) RIA. Graph shows the parallelism of serial dilutions of goldfish serum with respect to the standards provided by the assay manufacturer. The slope of the serial dilutions of the goldfish serum was not significantly different from the standards provided with the assay kit.

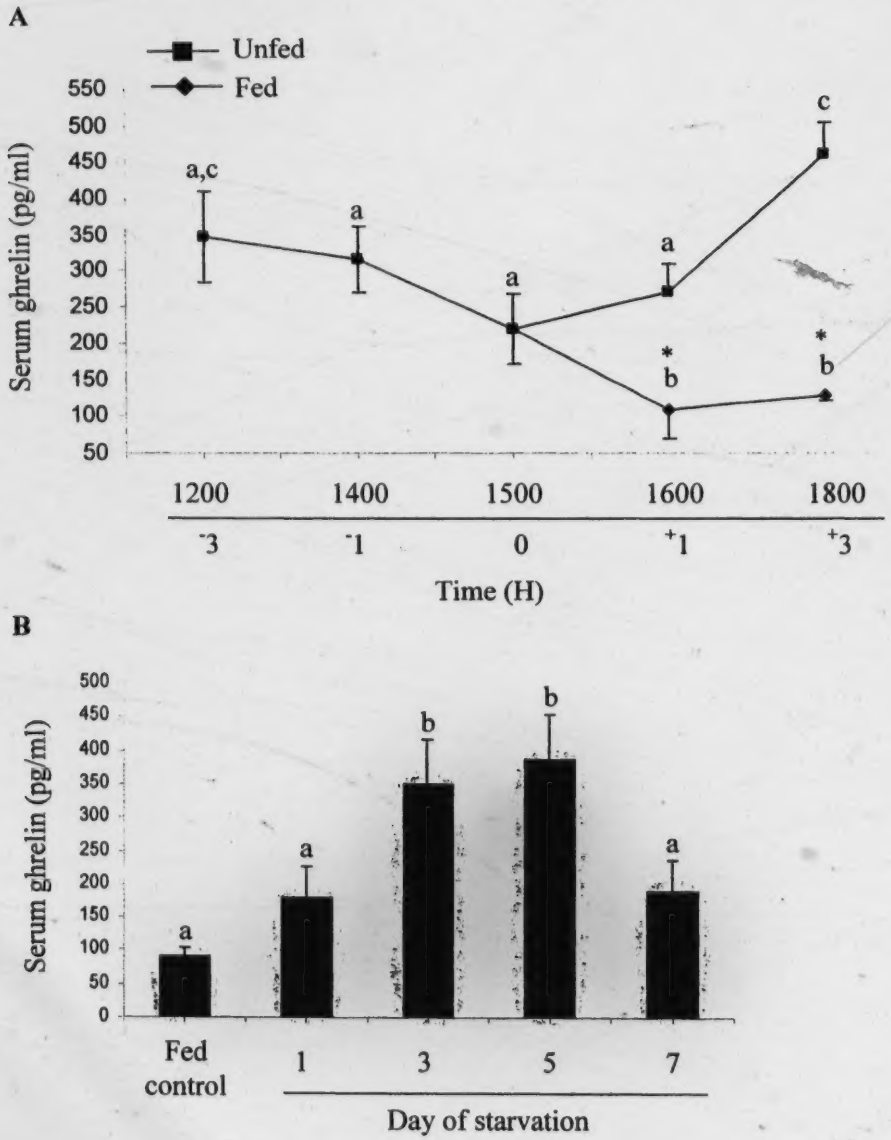
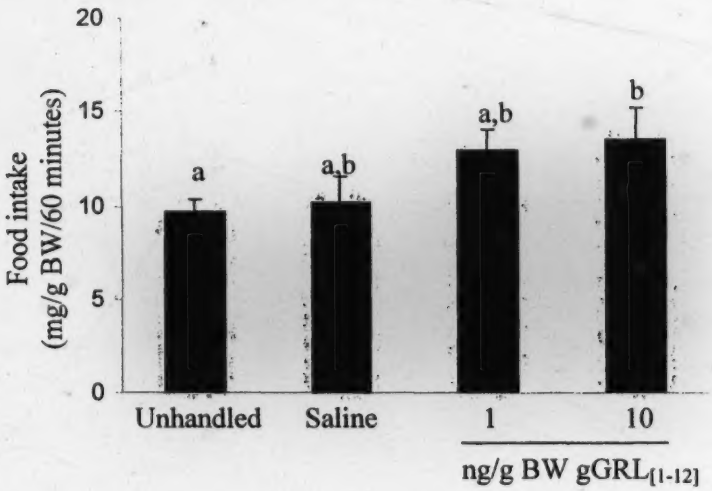


Figure 3.5. Preprandial and postprandial (A) and starvation induced (B) changes in serum ghrelin levels in goldfish. Same letters denote values that are not statistically different. Data is presented as mean±SEM. n = 7. Asterisks represent significant differences between groups at the same time point.

A Effects of ICV $gGRL_{[12]}$ on food intake



B Effects of ICV hGRL on food intake

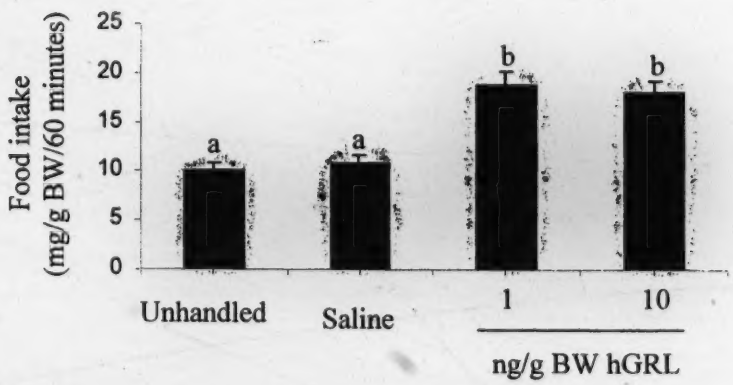
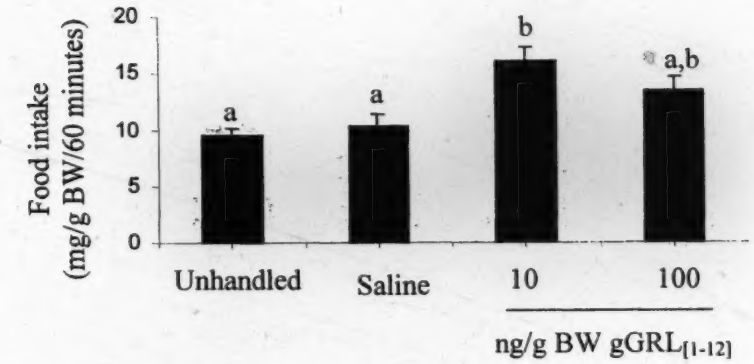


Figure 3.6. Effects of intracereboventricular (ICV) injections of $gGRL_{[1-12]}$ (A) and human ghrelin (hGRL) (B) on food intake in goldfish. Data is presented as mean±SEM. n = 8. Same letters denote values that are not statistically different.

A Effects of IP $gGRL_{[12]}$ on food intake



B Effects of IP $gGRL_{[19]}$ on food intake

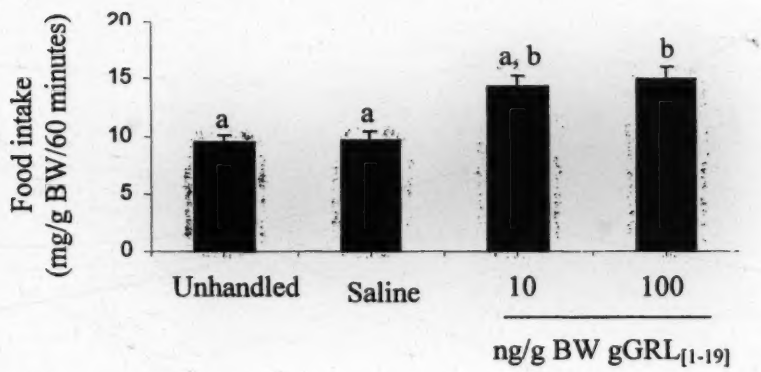


Figure 3.7. Effects of intraperitoneal (IP) injections of $gGRL_{[1-12]}$ (A) and $gGRL_{[1-19]}$ (B) on food intake in goldfish. Data is presented as mean±SEM. n = 8. Same letters denote values that are not statistically different.

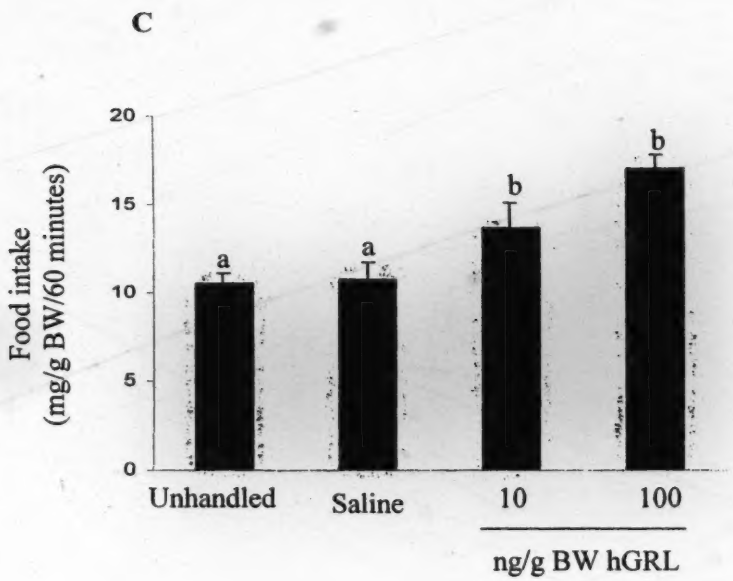


Figure 3.7.C Effects of intraperitoneal (IP) injections of human ghrelin (hGRL) on food intake in goldfish. Data is presented as mean±SEM. n = 8. Same letters denote values that are not statistically different.

CHAPTER 4

IN VITRO AND *IN VIVO* EFFECTS OF GHRELIN ON GROWTH HORMONE AND LUTEINIZING HORMONE RELEASE IN GOLDFISH

4.1. Introduction

Growth hormone secretagogues (GHS) are a group of peptide and non-peptide synthetic compounds that have potent growth hormone (GH) releasing activity (Tannenbaum and Bowers, 2001). In 1996, a G-protein coupled receptor to which the GHS can bind and elicit a biological response was identified from the pituitary and hypothalamus of humans and swine (Howard et al., 1996). This receptor has been named as the growth hormone secretagogue receptor (GHS-R) (Howard et al., 1996). In mammals, ghrelin is a multifunctional hormone, mainly involved in the regulation of growth hormone secretion (Kojima et al., 1999; Wren et al., 2000) and food intake (Tschop et al., 2000; Nakazato et al., 2001). Ghrelin stimulates GH release from rat (Kojima et al., 1999; Pinilla et al., 2003), porcine (Glavaski-Joksimovic et al., 2003) and bovine (Hashizume et al., 2003) somatotropes *in vitro*. Intracerebroventricular (ICV) (Date et al., 2000) and intraperitoneal (IP) (Wong et al., 1998) injections of ghrelin in rats, and intravenous (IV) injections in humans (Takaya et al., 2000) and rats (Thompson et al., 2003; Okimura et al., 2003) increase circulating GH levels.

A version of this chapter has been submitted for publication. Unniappan S and Peter RE 2003 *In Vitro* and *In Vivo* Effects of Ghrelin on Growth Hormone and Luteinizing Hormone Release in Goldfish. ***American Journal of Physiology Regulatory Integrative and Comparative Physiology.***

Recently, the structure of the mRNA encoding ghrelin in the goldfish has been reported (Chapter 2). There are two putative cleavage sites and amidation signals in the mature peptide region, suggesting the presence of two amidated ghrelin peptides of twelve ($gGRL_{[12]}$) and nineteen ($gGRL_{[19]}$) amino acids in length in the goldfish (Chapter 2). Although we have not purified the ghrelin peptides from goldfish, in a similar study, it was found that two peptides, orexin-A and orexin-B, originate from the prepro-orexin mRNA that has two cleavage sites and amidation signals (Sakurai et al., 1999). The seven amino acids in the N-terminal region of ghrelin are GSSFLSP in all species from which ghrelin has been identified, except the bullfrog [GLTFLSP] (Kaiya et al., 2001) and goldfish (Chapter 2). Strong expression of ghrelin mRNA was found in the gut and spleen, while weaker expression was also found in the brain of goldfish (Chapter 2).

Ghrelin has also been identified from other non-mammalian vertebrates, including the chicken (Kaiya et al., 2002), bullfrog (Kaiya et al., 2001), frog (Galas et al., 2002), eel (Kaiya et al., 2003a), tilapia (Kaiya et al., 2003b; Parhar et al., 2003) and the rainbow trout (Kaiya et al., 2003c). In bullfrog, chicken, eel and trout, more than one form of ghrelin was found. In rainbow trout, both amidated and non-amidated ghrelin, modified with octanoyl or decanoyl groups are present (Kaiya et al., 2003c). The role of the amide group in fish ghrelin is currently not known (Kaiya et al., 2003c). Octanoylated ghrelin is present in all species from which ghrelin has been isolated and identified, including the species in which ghrelin with other acyl modifications (eg: decanoylated ghrelin) were also found (Kaiya et al.,

2002; Kaiya et al., 2003c). ICV injections of octanoylated $gGRL_{[19]}$ stimulated food intake in goldfish (Chapter 2). We have detected a meal related change in octanoylated ghrelin in the goldfish serum using an assay specific for the n-octanoylated ghrelin (Unniappan et al., unpublished observations). Ghrelin stimulates GH and prolactin (PRL) release from incubated whole pituitaries of eel (Kaiya et al., 2003a) and tilapia (Riley et al., 2002; Kaiya et al., 2003b). Recently, Kaiya et al. (2003c) found that ghrelin stimulates GH release from incubated whole pituitaries of trout, while no effect was found on somatolactin and PRL release. Intracranial injections of ghrelin inhibited water intake in eels (Kozaka et al., 2003). In this chapter, the effects of ghrelin on GH and LH (recently a consensus had been reached to use the terms, "LH" for fish gonadotropin-II [GtH-II] and "LHβ" for fish "GtH-IIβ subunit" [Yaron et al., 2003]) release *in vitro* from dispersed pituitary cells and *in vivo* following ICV or IP injections in the goldfish are presented. The effects of ghrelin on GH and LHβ mRNA expression are also discussed. Further, the *in vitro* effects of somatostatin-14 (SS-14) on ghrelin induced GH release and the *in vitro* effects of the GHS-R antagonist, D-Lys^3-GHRP-6 on ghrelin induced GH and LH release in the goldfish is also presented.

4.2. Materials and Methods

Animals

Goldfish (*Carassius auratus*) of the common or comet varieties and approximately 35-45 g body weight undergoing a sexually regressed stage, were purchased from the Mount Parnell Fisheries (Mercersburg, Pennsylvania, USA) and

maintained under a simulated natural photoperiod (July/August) of Edmonton, Alberta, Canada. Fish used for the *in vitro* studies were maintained in a 300 l flow-through aquaria that received a constant flow of aerated and dechlorinated city water at 17 C, while the fish used for the *in vivo* experiments were kept in 65 l glass aquaria receiving aerated water at 20 C. Fish were fed daily at a specific time with commercially prepared fish pellets (Corey Aquafeeds, Corey Fish Mills Ltd., Fredericton, New Brunswick, Canada). Fish were acclimatized to the respective tanks for a minimum of 14 days. All protocols were approved by the Faculty of Sciences Animal Care Committee, University of Alberta, based on the guidelines of the Canadian Council for Animal Care.

Hormones and receptor antagonist

$gGRL_{[1-12]}$ and $gGRL_{[1-19]}$ with n-octanoyl modification (Chapter 2) were synthesized in the laboratory of Dr. Jean E. Rivier, Clayton Foundation Laboratories for Peptide Biology, The Salk Institute of Biological Sciences (San Diego, California, USA). Although goldfish ghrelin have not been isolated and characterized, the synthetic octanoylated forms of both putative peptides ($gGRL_{[1-12]}$ and $gGRL_{[1-19]}$) were used in the present study. Ghrelin stocks were made by dissolving the peptide in sterile, double distilled water. Stocks were kept at ¯20 C. Peptide stocks were thawed and then diluted in fish physiological saline (Burnstock, 1958) for *in vivo* studies or in testing media for *in vitro* studies. D-Lys^3-GHRP-6 was purchased from Phoenix Pharmaceuticals (Belmont, California, USA). SS-14 was purchased from Peninsula (Bachem) Laboratories (San Carlos, California,

USA). SS-14 and D-Lys^3-GHRP-6 stocks were made by dissolving in sterile double distilled water and frozen. The stocks were diluted in testing media for experiments. Thawed peptide stocks were never refrozen.

Tissue culture reagents

Medium 199 was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Bovine serum albumin (BSA, fraction V) was purchased from Calbiochem (EMD Biosciences Inc., San Diego, California, USA). All other solutions and media used for pituitary dispersion, perifusion and static incubation experiments and primers for amplifying the GH and LHβ probes were purchased from the Gibco-Invitrogen Life Technologies (Burlington, Ontario, Canada).

In vitro experiments

Dispersion of pituitary cells

Fish were anesthetized by submersion in 0.05% tricaine methanesulfonate (MS-222, Syndel Laboratories, Vancouver, British Columbia, Canada), euthanized and pituitaries were removed and placed in dispersion media (Medium 199 with Hank's salts, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 0.3% BSA, 100 000 U/l penicillin, 1000 mg/l streptomycin, pH adjusted to 7.18 with NaOH). Pituitary cells were dispersed by trypsin/DNase treatment (modified protocol from Chang et al., 1990) and cells were resuspended in plating medium (which has the same composition of the dispersion medium except that Medium 199 with Earle's salts was used and the 0.3% BSA was substituted with 1% horse serum).

Column perifusion of pituitary cells

Perifusion experiments were conducted as described earlier (modified protocol from Chang et al., 1990). Briefly, dispersed cells were cultured overnight on preswollen cytodex beads at 28 C and 5% CO_2 humidity. Prior to the experiment, approximately 1.5 x 10^6 dispersed cells were loaded onto each column and perifused with testing medium (Medium 199 with Hank's salts, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 0.1% BSA, 100 000 U/l penicillin, 1000 mg/l streptomycin, pH adjusted to 7.18 with NaOH) for 4 h at a rate of 15 ml/h to stabilize the basal secretion rate of GH and LH. Following the initial 4 h perifusion, six, 5 minute fractions were collected. The average GH concentration in these fractions was considered as the basal hormone release level. On the seventh fraction, a 5 minute pulse of testing media containing the specified concentration (0.001, 0.01, 0.1, 1 and 10 nM) of $gGRL_{[1-12]}$ or $gGRL_{[1-19]}$ was administered and the perifusion media was switched back to the normal testing media and 5-13 more five minute fractions collected (a total of 12-25 fractions from each column). The perifusate fractions were stored at -20 C until the GH and LH radioimmunoassay (RIA). Only a single dose of $gGRL_{[1-12]}$ or $gGRL_{[1-19]}$ was applied to each column.

The effect of SS-14 on ghrelin's stimulatory role on GH release was studied using column perifusion. In this experiment, following the collection of six, 5 minute fractions, in 4 perifusion columns the perifusion media was changed to testing media containing 100 nM of SS-14, while 2 perifusion columns were

continuously perifused with plain testing media (control columns). After 30 min (6 fractions), a 5 minute pulse of 10 nM $gGRL_{[19]}$ was administered to all columns and perifusion was continued with media containing SS-14 or plain testing media. After another 25 min (5 fractions), the medium was switched back to plain testing media in the four columns perifused with SS-14. A total of 24 fractions were collected from each column. The perifusate fractions were stored at ¯20 C until the GH and LH RIA.

Static incubation of dispersed pituitary cells

For the 2 h static incubation experiments on GH release, 0.25×10^6 cells/ml/well were cultured overnight in plating medium on 24 well BD Falcon Primaria culture plates (BD Biosciences, Mississauga, Ontario, Canada) under 5% CO_2 humidity and 28 C as described previously (modified protocol from Chang et al., 1990). Prior to the experiment, the wells were emptied, 1 ml of fresh testing medium was added and the plates were again incubated under the same conditions. After 1 h incubation, plates were taken out, and depending on the experiment, 1 ml of plain testing media or testing media containing 1 or 10 nM of $gGRL_{[1-12]}$ or $gGRL_{[1-19]}$, 10 nM $gGRL_{[1-19]}$ and 1000 nM SS-14 or 10 nM $gGRL_{[19]}$ and 1000 nM D-Lys^3-GHRP-6 were added and the plates were incubated again under the same conditions. After 2 h, 800µl of the media was collected from each well and stored at ¯20 C until the RIA for GH and LH. All doses were tested in a minimum of 4 wells in each experiment and the experiments were repeated at least three times.

mRNA expression studies

Static culture and total RNA extraction

In this experiment, approximately 2 x 10^6 cells/ml/well were plated. On the day of the experiment, the wells were emptied and 1 ml of new testing media was added and the plates incubated for 1 h. The testing media was removed and plain testing media, or testing media containing 1 or 10 nM of $gGRL_{[12]}$ or $gGRL_{[19]}$ was added and the plates incubated under the same culture conditions described above. After 2 h, 800µl of the media was removed and 1 ml of TRIzol reagent (Gibco-Invitrogen) was added, mixed by repeated pipeting and the whole contents of each well transferred to a RNase free microfuge tube kept on wet ice. Total RNA was extracted immediately following the manufacturer's (TRIzol; Gibco-Invitrogen) protocol. The concentration of the RNA was determined using a spectrophotometer at 260/280 nm. The RNA samples were kept at $^{-}$80 C until the slot blot analysis was carried out.

Slot blot analysis of GH and LH mRNA expression

2 µg of total RNA was blotted on to a Hybond Nylon membrane (Amersham Biosciences, Baie d'Urfe, Quebec, Canada) using the Bio-Dot SF blotting apparatus (Bio-Rad Laboratories, Hercules, California, USA). Specific cDNA probes covering the full length of the goldfish LHβ subunit cDNA (GenBank accession number D88024) and 716 bp of the goldfish growth hormone cDNA (GenBank Accession number AF401272) were amplified using specific primers, GtH-F (5'ACTTTTAACAGCCTGCTGAG3') and GtH-R (5'ACATTTACACAA

CATTTATT3') for LHβ, and GH-F (5'ATGGCTAGAGCATTAGTGCTG3') and GH-R (5'GATCATAATAACTTAAGGAAG3') for GH. The PCR conditions for amplifying LHβ cDNA probes were denaturation at 94 C for 5 min followed by 35 cycles of denaturation for 1 min at 95 C, annealing at 54 C for 1 min and extension at 73 C for 1 min. The PCR conditions for amplifying the GH probe were denaturation at 95 C for 5 min followed by 25 cycles of denaturation for 1 min at 95 C, annealing for 1 min at 50 C and extension for 1 min at 72 C and a final extension at 72 C for 5 min. The probes were labeled with [α-^{32}P]dCTP using a Rediprime II – Quickprime kit (Qiagen Inc., Mississauga, Ontario, Canada) following the manufacturer's protocol. The labeled probes were then purified using a QIAquick nucleotide removal kit (Qiagen) following the manufacturer's instructions. 10^6 cpm/ml of probe were used for hybridization. First, the membrane was hybridized with the GH probe using previously described methods (Chapter 2). After hybridization overnight at 65 C, the membranes were washed twice with washing solution (0.04 M $NaHPO_4$, 1mM EDTA, and 1% SDS) at 65 C. The membranes were then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 3 hs and the screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). The membrane was then stripped and reprobed with the LH-β probe as described previously and exposed to a phosphor screen for 4 hs. The membrane was then stripped again and reprobed with an [α-^{32}P]dCTP labeled goldfish β-actin (GenBank accession number AF079831) probe as an internal control. In order to reduce

variability, the control and experimental samples were blotted on the same nylon membrane.

In vivo experiments

ICV injections

Our previous studies found that ICV injections of 1 ng/g bodyweight of $gGRL_{[19]}$ stimulated food intake in the goldfish (Chapter 2). Hence, we selected this dose for ICV injections in the current study. Four groups (for sampling at 15, 30, 45 or 60 min) of 12 fish (6 control fish and 6 experimental fish) each were acclimatized to the aquarium conditions for at least 14 days. ICV injections were conducted as described earlier (Chapter 2). Briefly, fish were anesthetized and a three-sided flap was cut in the roof of the skull using a circular saw attached to a dentist drill. The flap was then folded to the side and the brain exposed. Fish were then placed in a stereotaxic apparatus and the needle of a 5 µl Hamilton microsyringe (Hamilton Co., Reno, Nevada, USA) was placed in the preoptic region of the brain according to the coordinates (+1.0, M, D 1.2) from the brain atlas of Peter and Gill (1975). At time zero (T = 0), fish were injected with 2 µl of fish physiological saline (control fish) or 2 µl of fish physiological saline containing 1 ng/g BW of $gGRL_{[19]}$ (experimental fish). The cranial cavity was then filled with fish physiological saline and the skull flap replaced and sutured using surgical thread. Fish were put back into their respective aquaria to recover from anesthesia (which normally occurs within 5 min). Six control fish and six experimental fish were anesthetized and blood samples were collected by caudal puncture at 15 min post-injection. Similarly, 6 control and 6

experimental fish were sampled at 30, 45 or 60 min post-injection. The experiments were conducted in two days. Injections were administered in a staggered manner to enable timely sampling. Each fish underwent ICV injections and blood sampling only once. Samples were kept at 4 C for several hs to allow clotting and then spun at 8000 rpm for 5 min to collect serum. Serum samples were stored at ¯20 C until the RIA for GH and LH.

IP injections

Previously, we found that IP injections of 100 ng/g bodyweight of $gGRL_{[19]}$ stimulated food intake in the goldfish (Unniappan et al., unpublished observations). Hence, we used 100 ng/g bodyweight of $gGRL_{[19]}$ for IP injections in the current study. Four groups (for sampling at 15, 30, 45 or 60 min) of 16 fish (8 control fish and 8 experimental fish) each were acclimatized to the aquarium conditions for at least 14 days. IP injections were conducted as previously described (Himick and Peter, 1994). Briefly, fish were anesthetized and placed in a wet sponge holder. At time zero (T = 0), one group of fish (n = 2) was injected with 100 µl of fish physiological saline (control fish), while a second group (n = 2) was injected with 100 µl of fish physiological saline containing 100 ng/g of $gGRL_{[19]}$ (experimental fish) in the peritoneal cavity using a 26 gauge needle (Beckton Dickinson Co., Franklin Lakes, New Jersey, USA) attached to a 250 µl Hamilton syringe. 8 control fish and 8 experimental fish were anesthetized and blood samples were collected by caudal puncture at 15 min post-injection. Similarly, 8 control or 8 experimental fish were sampled at 30, 45 or 60 min post-injection. The experiments were conducted in

2 days. Injections were administered in a staggered manner to enable timely sampling. Each fish underwent IP injections and blood sampling only once. Samples were kept at 4 C for several hs to allow clotting and then spun at 8000 rpm for 5 min to collect serum. Serum samples were stored at ¯20 C until the RIA for GH (see Marchant et al., 1989) and LH (see Peter et al., 1984).

Hormone measurements

All samples were assayed for both GH and LH (except the SS experiment samples that were assayed only for GH). GH and LH levels in the perifusate fractions, static culture samples and serum samples were measured using previously validated RIAs for GH (see Marchant et al., 1989) and LH (see Peter et al., 1984). The GH RIA detects both forms of growth hormone in goldfish (Chang JP, personal communication). All samples were assayed in duplicate in the same RIA.

Data analysis

Data from the perifusion experiments are presented as the percentage of the prepulse GH or LH levels ± SEM. Prepulse GH or LH levels is the average of the GH or LH content in the first 6 fractions of perifusate collected before starting the administration of the hormone. Since the basal levels of GH in different columns varied, data were first converted to the percentage basal of individual columns and then pooled together. The fraction that shows an stimulation (peak) in GH or LH release, after the administration of the peptide was taken and the percentage increase, compared to the prepulse was calculated. For static incubation experiments, the data are presented as the percentage of GH or LH levels in control wells ± SEM. The GH

and LH mRNA levels were expressed as a ratio of the GH or LHβ mRNA to β actin mRNA, and then normalized as a percentage of its expression levels in the cells in control wells. Statistical analysis of data from the perifusion and static incubation experiments was conducted by ANOVA followed by Least Significant Difference test. Data from the *in vivo* experiments (ICV and IP injections) was analyzed using Independent T-test. Significance was considered at $p < 0.05$.

4.3. Results

Cell column perifusion

0.01, 0.1, 1 and 10 nM $gGRL_{[12]}$ caused a significant increase in GH release (Figure 4.1.A). The effects of 10 nM $gGRL_{[12]}$ in stimulating GH release were significantly greater than the other doses tested. Doses of 0.1, 1 and 10 nM $gGRL_{[12]}$ caused a significant stimulation of LH release (Figure 4.1.B); however, there were no significant differences in LH release elicited by these dosages of $gGRL_{[12]}$. No significant effects were found for 0.001nM $gGRL_{[12]}$ on GH (Figure 4.1.A) release and 0.001nM and 0.01 nM $gGRL_{[12]}$ on LH release (Figure 4.1.B).

0.01, 0.1, 1 and 10 nM $gGRL_{[19]}$ caused a significant stimulation of both GH (Figure 4.2.A) and LH release (Figure 4.2.B). There were no differences in GH or LH release responses elicited by 0.01, 0.1, 1 and 10 nM $gGRL_{[19]}$ different concentrations of $gGRL_{[19]}$ tested. No significant stimulatory effects were found for 0.001nM $gGRL_{[19]}$ on GH (Figure 4.2.A) and LH release (Figure 4.2.B).

100 nM of SS-14 inhibited the stimulatory effects of 10 $gGRL_{[19]}$ on GH release from perifused pituitary cells of goldfish (Figure 4.3).

Static incubation experiments

10 nM of $gGRL_{[12]}$ stimulated both GH (Figure 4.4.A) and LH (Figure 4.4.B) release from dispersed pituitary cells in static culture. Both 1 and 10 nM $gGRL_{[19]}$ stimulated GH release (Figure 4.5.A), while only 10 nM $gGRL_{[19]}$ was effective in stimulating LH release (Figure 4.5.B). No effects were found for 1 nM $gGRL_{[12]}$ on GH and LH release and 1 nM $gGRL_{[19]}$ on LH release.

SS -14 inhibited 10 nM $gGRL_{[19]}$ induced GH release from dispersed pituitary cells in static culture (Figure 4.6). GHS-R antagonist, D-Lys^3-GHRP-6 had no effect on the stimulatory effects of 10 nM $gGRL_{[19]}$ on GH release from dispersed pituitary cells in static culture (Figure 4.7.A). D-Lys^3-GHRP-6 inhibited ghrelin's stimulatory effect on LH release from dispersed pituitary cells in static culture (Figure 4.7.B).

GH and LHβ mRNA expression

10 nM of $gGRL_{[19]}$ increased GH mRNA expression in dispersed pituitary cells in static culture (Figure 4.8.A). 10 nM of $gGRL_{[12]}$ and 1 and 10 nM of $gGRL_{[19]}$ stimulated LHβ mRNA expression in dispersed pituitary cells in static culture (Figure 4.8.B). Stimulatory effects of 10 nM $gGRL_{[19]}$ on LHβ mRNA expression were higher than that of 10 nM $gGRL_{[12]}$ and 1 nM $gGRL_{[19]}$. No significant stimulatory effect was found for 1 nM $gGRL_{[12]}$ on LHβ mRNA expression (Figure 4.8.B). No stimulatory effects were found for 1 nM and 10 nM of $gGRL_{[12]}$ and 1 nM of $gGRL_{[19]}$ on GH mRNA expression (Figure 4.8.A).

ICV injections

ICV administration of 1 ng/g BW of $gGRL_{[19]}$ caused a significant increase in serum GH levels at 15 and 30 min post-injection (Figure 4.9.A). Serum GH levels at 45 and 60 min post-injection were not significantly different from the saline injected controls (Figure 4.9.A). ICV administration of 1 ng/g BW of $gGRL_{[19]}$ significantly increased serum LH levels at 60 min post-injection (Figure 4.9.B). No significant changes in serum LH levels were observed at 15, 30 or 45 min post-injection (Figure 4.9.B).

IP injections

IP administration of 100 ng/g BW of $gGRL_{[19]}$ caused a significant increase in serum GH levels in goldfish at 15 and 30 min post-injection (Figure 4.10.A). No significant in serum GH levels were found at 45 and 60 minute post-injection (Figure 4.10.A). IP administration of 100 ng/g BW of $gGRL_{[19]}$ significantly increased serum LH levels 60 min post-injection (Figure 4.10.B). No significant differences in serum LH levels were found at 15, 30, and 45 min post-injection (Figure 4.10.B).

4.5. Discussion

Effects of ghrelin on LH and GH release

A novel finding of this study is the *in vitro* and *in vivo* stimulation of LH release in goldfish by $gGRL_{[1-12]}$ and $gGRL_{[1-19]}$. Both peptides dose dependently stimulated LH release from perifused pituitary cells and pituitary cells in static culture. These results imply that ghrelin acts directly on the goldfish gonadotropes. The lowest dose that stimulates LH release from the goldfish pituitary cells is 0.01

nM of $gGRL_{[1-19]}$. The minimal effective dose of ghrelin in this study is a dose in between 0.001 nM and 0.01 nM of $gGRL_{[1-19]}$ or a dose between 0.01 nM and 0.1 nM of $gGRL_{[1-12]}$, indicating that ghrelin is a potent LH releasing hormone in goldfish. The minimal effective doses of ghrelin that stimulates LH release are comparable to the minimal effective doses of other LH releasing hormones in goldfish, such as the gonadotropin-releasing-hormone (GnRH, Marchant et al., 1989) and neuropeptide Y (NPY, Peng et al., 1993). These results indicate that ghrelin acts directly on the goldfish gonadotropes. In a preliminary study, it was found that human ghrelin also stimulates LH release from dispersed pituitary cells *in vitro* (Unniappan et al., unpublished observations), adding more supportive evidence for our current findings. The results presented above contradict a previous report that found no effects of ghrelin on LH and FSH release *in vitro* from the pituitary cells of rats (Kojima et al., 1999).

As the *in vitro* studies indicated that both $gGRL_{[1-12]}$ and $gGRL_{[1-19]}$ are biologically active in stimulating LH release, $gGRL_{[1-19]}$ was used for the *in vivo* studies . Both ICV (1ng/g) and IP (100 ng/g) injections of $gGRL_{[19]}$ significantly increased serum LH levels at 60 min post-injection. These results agree with the findings of the *in vitro* studies. However, no effects of ghrelin on LH release *in vivo* in rats were found (Kojima et al., 1999; Tolle et al., 2001). In sheep, ICV injection of ghrelin decreases LH pulse frequency, but not amplitude (Furuta et al., 2001). The results indicating the stimulation of LH release *in vitro* and *in vivo* by ghrelin in goldfish are unique. The results of the present study add to a list of functions (eg:

LH releasing activity) in certain animal groups or species. For example, ghrelin is an orexigen in mammals (Tschop et al., 2000; Nakazato et al., 2001) and in goldfish (Chapter 2), while it is a potent anorexigen in chicken (Saito et al., 2002). Similarly, ghrelin stimulates PRL release in tilapia (Riley et al., 2002) and eel (Kaiya et al., 2003a), while it has no effects on PRL release in the rainbow trout (Kaiya et al., 2003c).

In the present study, it was found that ghrelin dose-dependently stimulates GH release from dispersed pituitary cells of goldfish in perifusion and static incubation. These results indicate that ghrelin acts directly on the goldfish somatotropes. The lowest dose that stimulates GH release from goldfish pituitary cells is 0.01 nM. The minimal effective dose of ghrelin in this study is a dose in between 0.001 nM and 0.01 nM, indicating that ghrelin is a potent GH releasing hormone in goldfish. A recent study found that 1 picomolar ghrelin is effective in stimulating GH release from porcine somatotropes *in vitro* (Malagon et al., 2003). In goldfish, zebrafish PACAP-38 (zPACAP-38) stimulates GH release from dispersed pituitary cells at doses as low as 0.01 nM (Wong et al., 1998). The minimal effective doses of ghrelin we found in this study are comparable to the minimal effective doses of ghrelin in mammals and zPACAP-38 in goldfish. In a preliminary study, we found that human ghrelin also stimulates GH release from dispersed pituitary cells *in vitro* (Unniappan et al., unpublished observations), adding more support for the current findings. Ghrelin stimulates GH release from the dispersed pituitary cells of rats (Kojima et al., 1999; Pinilla et al., 2003), pigs (10), and cattle (11).

Stimulatory effects of ghrelin on GH release were also reported from studies using the incubated whole pituitaries of tilapia (Riley et al., 2002; Kaiya et al., 2003b), eel (Kaiya et al., 2003a) and trout (Kaiya et al., 2003c). Our results agree with these studies that found a stimulatory effect for ghrelin on GH release in mammals and fish.

Both ICV (1ng/g) and IP (100 ng/g) injections of $gGRL_{[19]}$ significantly increased serum GH levels. These results support our *in vitro* studies that found a stimulatory effect for ghrelin on GH release. In the present study, both ICV and IP injections of $gGRL_{[19]}$ significantly elevated serum GH levels 15-30 min post-injection. By 45-60 min post-injection, serum GH levels returned to normal levels. Single IP injection of the synthetic GHS, KP-102, stimulates GH release *in vivo* in tilapia after 6 h and 12 h post-injection (Shepherd et al., 2000). Recently, it was reported that IP injections of 250 ng/g bodyweight of trout ghrelin increased serum GH levels in rainbow trout 30-60 min post-injection, while no effects were found on prolactin and somatolactin levels (Kaiya et al., 2003c). Ghrelin has been demonstrated to stimulate GH release *in vivo* in rats (Date et al., 2000; Wren et al., 2000; Tolle et al., 2001), chicken (Ahmed and Harvey, 2002; Kaiya et al., 2002) and rainbow trout (Kaiya et al., 2003c). Our results agree with the results of these studies on other vertebrate species.

The mechanisms of action of ICV administered ghrelin on GH and LH release are currently not known. In goldfish, the pituitary is directly innervated by hypothalamic neurons (Fryer and Maler, 1981). It is possible that the ICV injected

ghrelin causes differential actions on the hypothalamic neurons expressing hypophysiotropic peptides (eg: SS, growth hormone releasing factor, GnRH) to stimulate GH or LH release in goldfish. It is also possible that ICV injected ghrelin might diffuse to the pituitary or get transported to the pituitary by the blood vasculature that anastomoses the third ventricle and the pituitary (Peter and Fryer, 1983).

Role of SS-14 on ghrelin induced GH release

The present study demonstrates that SS-14 can inhibit the stimulatory effects of ghrelin on GH secretion *in vitro*. SS-14 inhibits both basal (Yunker et al., 2003) and stimulated GH release *in vitro* (Peng et al., 1993) in the goldfish. The inhibitory actions of SS-14 on ghrelin stimulated GH release provide further evidence for ghrelin's direct actions on goldfish pituitary cells. Our results agree with the previous reports on SS's effects on the GH releasing activity of ghrelin in mammals. SS or SS analogues inhibit the *in vivo* effects of ghrelin on GH release in humans (Broglio et al., 2002) and rats (Tannenbaum et al., 2003) and its *in vitro* GH releasing effects from cultured porcine pituitary cells (Hashizume et al., 2003). Several unpublished observations from our laboratory and similar studies conducted in other cyprinid fish (Lin et al., 1993) found no effects of SS on LH release.

Role of GHS-R antagonist on ghrelin induced GH and LH release

GHS-R1a is the only known active ghrelin receptor (Howard et al., 1996). D-Lys^3-GHRP-6 is a specific GHS-R antagonist used in studies on the effects of ghrelin on GH release in mammals (Glavaski-Joksimovic et al., 2003; Okimura et

al., 2003). Interestingly, D-Lys^3-GHRP-6 inhibited the stimulatory effects of 10 nM $gGRL_{[19]}$ on LH release, while no effects were found on the stimulatory effects of 10 nM $gGRL_{[19]}$ on GH release from goldfish pituitary cells in static culture. These results indicate that gonadotropes in goldfish have a GHS-R1a-like receptor, whereas somatotropes have a different GHS-R. Chen (2000) proposed the presence of more than one receptor in mediating the effects of synthetic GHS. Recently, Thompson et al. (2003) found that ghrelin stimulates bone marrow adipogenesis *in vivo* in rats by acting independent of the GHS-R1a, suggesting the presence of a receptor other than the GHS-R1a in mediating the physiological functions of ghrelin in rats. It is also worth mentioning that D-Lys^3-GHRP-6 only decreased the ghrelin-induced increase in intracellular calcium concentration in porcine somatotropes (Glavaski-Joksimovic et al., 2003), and had no effects on ghrelin- stimulated GH release *in vivo* in rats (Okimura et al., 2003). GHS-R has been identified in the pufferfish, *Spheroides nephelus* (Palyha et al., 2000). Pufferfish GHS-R has a high structural similarity with the mammalian GHS-Rs, suggesting that the receptor structure and function are evolutionarily conserved (Palyha et al., 2000). The structure and distribution of goldfish GHS-R or related receptors are currently unknown.

In vitro effects of ghrelin on GH and LHβ mRNA

Only 10 nM $gGRL_{[19]}$ stimulated GH mRNA expression, while 10 nM $gGRL_{[1-12]}$ and both 1 and 10 nM $gGRL_{[1-19]}$ stimulated LHβ mRNA expression. $gGRL_{[19]}$ elicited a dose dependent increase in LHβ mRNA expression. These results indicate that in addition to the stimulation of GH and LH release, ghrelin also

possibly stimulates the synthesis of GH and LH in the goldfish. Previously, it has been shown that releaser hormones such as GnRH stimulate both GH and LHβ mRNA expression in the goldfish pituitary cells *in vitro* (Klausen et al., 2001). However, in the only study available on the effects of ghrelin on GH mRNA expression in mammals, ICV injections of ghrelin had no effects on GH mRNA expression in rat pituitary (Date et al., 2000). The basis for the difference in the effects of ghrelin on GH mRNA and LHβ mRNA expression may be due to various factors including the presence of multiple receptors mediating the actions of ghrelin and the differential effects on gene expression by signaling cascades in the different cell types. In the present study, we found that GHS-R antagonist blocks the effects of ghrelin on LH release, while no effects were found on GH release, indicating that ghrelin acts on LH and GH release through different receptors. It is also clear that the effects of regulatory peptides on LH subunit mRNA expression are differentially transduced by various intracellular signaling mechanisms (Yaron et al., 2003).

In conclusion, we provide evidence for the LH- and GH- releasing activity of $gGRL_{[12]}$ and $gGRL_{[19]}$ in goldfish. Ghrelin acts directly on goldfish pituitary cells to release GH and LH. Both central and peripheral injections of ghrelin stimulate LH and GH release *in vivo*. Ghrelin also stimulates LHβ and GH mRNA expression, indicating that ghrelin is also involved in the synthesis of LH and GH in goldfish. SS-14 abolishes ghrelin's stimulatory effects on GH secretion. GHS-R antagonist did not inhibit ghrelin's stimulatory effects on GH, while it completely abolished the effects of ghrelin on LH release, indicating the presence of more than one receptor in

mediating the hypophysiotropic actions of ghrelin. The results of the present study, together with our previous reports on ghrelin's stimulatory role on food intake, indicate that ghrelin is a hypophysiotropic orexigen, linking the physiology of food intake, growth and reproduction in goldfish.

4.5. REFERENCES

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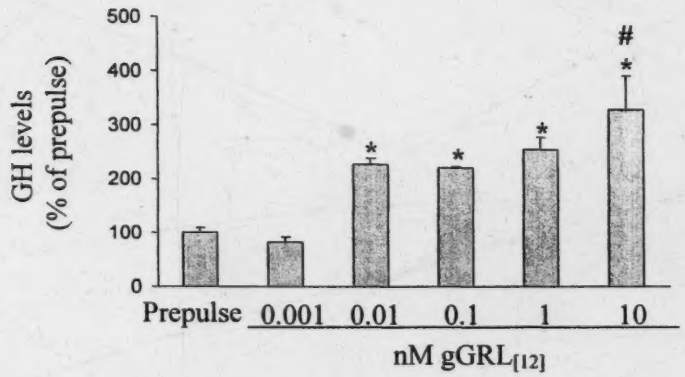
A. Effects of $gGRL_{[12]}$ on GH release from perifused pituitary cells



B. Effects of $gGRL_{[12]}$ on LH release from perifused pituitary cells

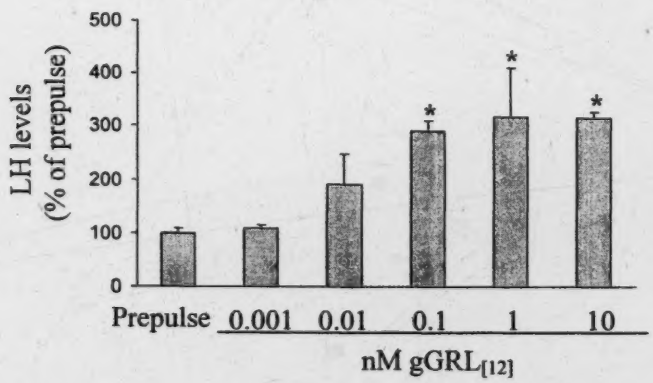
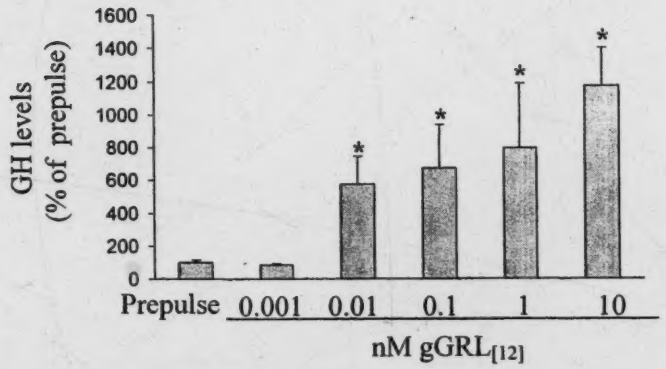


Figure 4.1. Effects of various concentrations of $gGRL_{[12]}$ on GH (A) and LH (B) release from the perifused pituitary cells of goldfish. Data is presented as mean±SEM. n = 4 columns (0.001, 0.01 and 0.1 nM) or 6 columns (1 and 10 nM) [pooled from two different experiments]. Results were analyzed using ANOVA followed by Least Significant Difference test. An asterisk (*) represents values that are significantly higher than the prepulse. The sign (#) represents the value that is significantly higher than the other treatment groups.

A. Effects of $gGRL_{[19]}$ on GH release from perifused pituitary cells



B. Effects of $gGRL_{[19]}$ on LH release from perifused pituitary cells

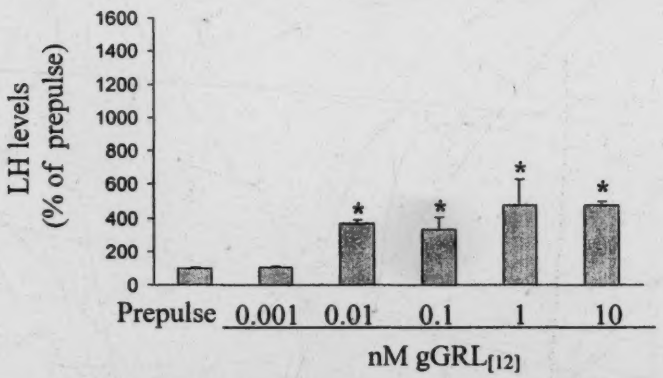


Figure 4.2. Effects of various concentrations of $gGRL_{[19]}$ on GH (A) and LH (B) release from the perifused pituitary cells of goldfish. Data is presented as mean±SEM. n = 4 columns (pooled from two different experiments). Results were analyzed using ANOVA followed by Least Significant Difference test. An asterisk (*) represents values that are significantly higher than the prepulse.

Effect of SS-14 on $gGRL_{[19]}$ induced GH release from perifused somatotropes

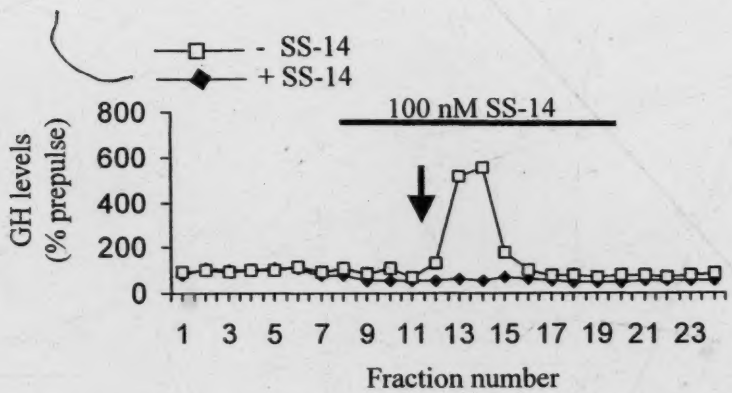
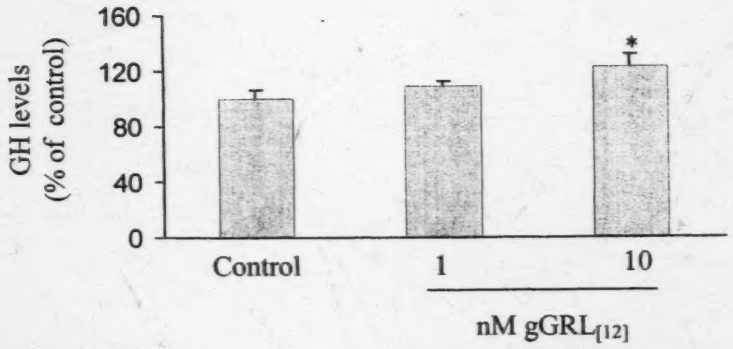


Figure 4.3. Effect of SS-14 on $gGRL_{[19]}$ induced GH release from dispersed pituitary cells in perifusion. The open squares represent the perifusion without SS-14 administration, while the black diamonds represent the perifusion with SS-14. The black bar represents the fractions (fraction number 7-19) that were perifused with 100 nM SS-14. The arrow head points to the fraction in which 10 nM of $gGRL_{[19]}$ was administered. Data is presented as mean±SEM. n = 4 columns (for SS-14) or 2 columns (positive control without SS-14) from a single experiment

A. Effects of $gGRL_{[12]}$ on GH release from pituitary cells in static culture



B. Effects of $gGRL_{[12]}$ on LH release from pituitary cells in static culture

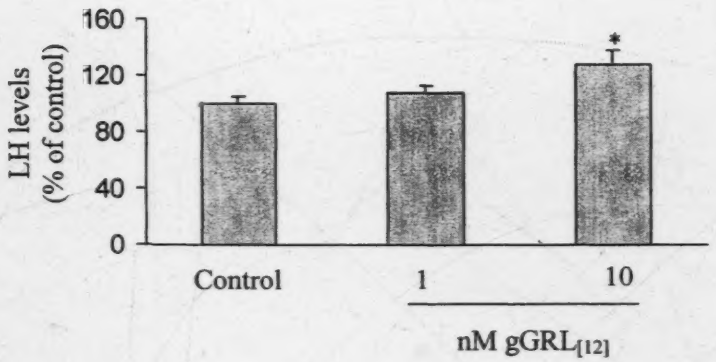
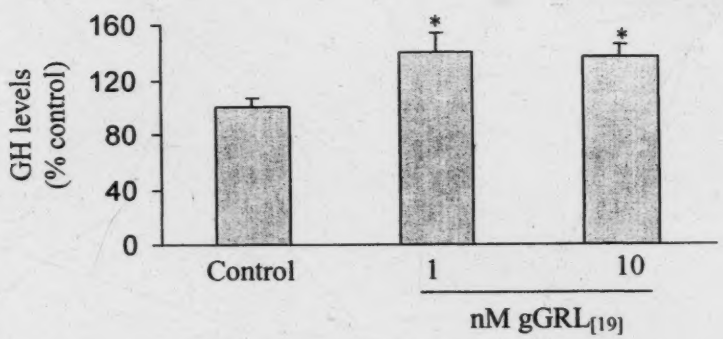


Figure 4.4. Effect of $gGRL_{[12]}$ on GH (A) and LH (B) release from dispersed pituitary cells in a 2 hour static culture. Data is presented as mean±SEM. n = 24 wells (pooled from 3 different experiments). Each treatment group was compared with the control group using Independent t-test. An asterisk (*) represents values that are significantly different from the control.

A. Effects of $gGRL_{[19]}$ on GH release from pituitary cells in static culture



B. Effects of $gGRL_{[19]}$ on LH release from pituitary cells in static culture

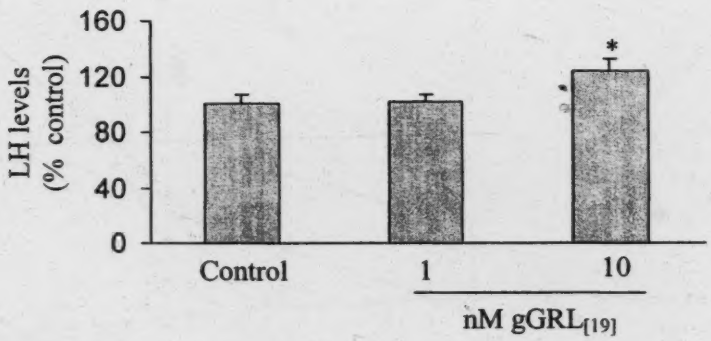


Figure 4.5. Effect of $gGRL_{[19]}$ on GH (A) and LH (B) release from dispersed pituitary cells in a 2 hour static culture. Data is presented as mean±SEM. n = 20 wells (pooled from 3 different experiments). Each treatment group was compared with the control group using Independent t-test. An asterisk (*) represents values that are significantly different from the control.

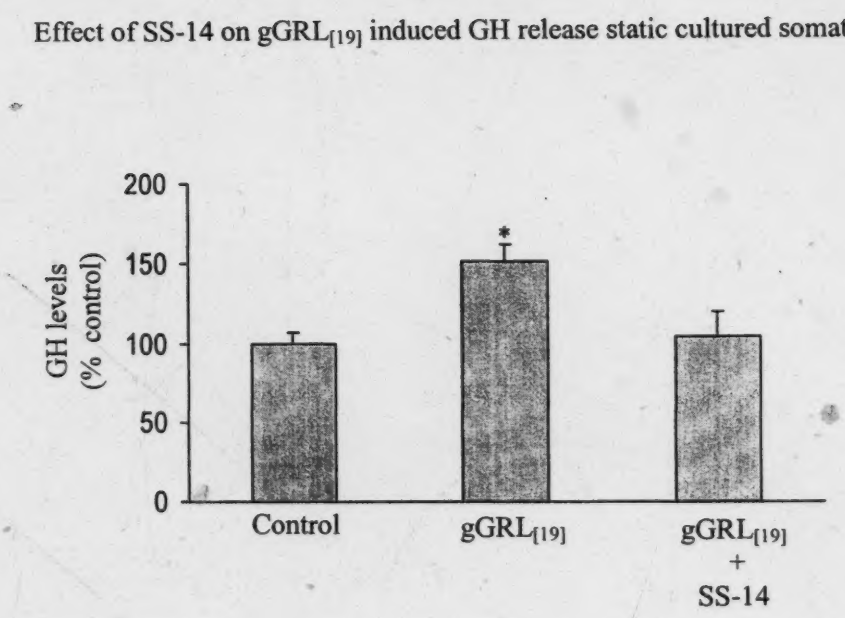
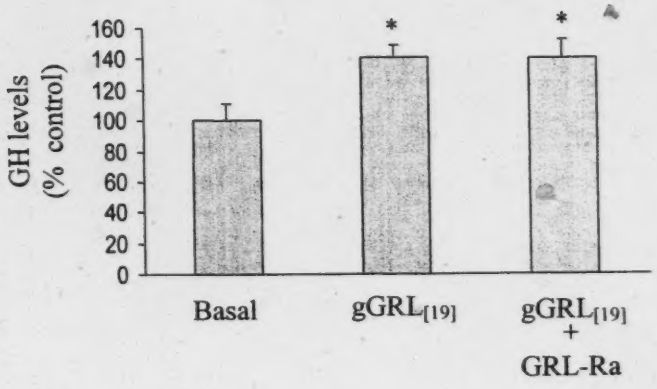


Figure 4.6. Effects of somatostatin on the ghrelin induced increase in GH release in static culture. The grey bars represent the GH levels in control (plain testing media), 10 nM $gGRL_{[19]}$ or 10 nM $gGRL_{[19]}$ + 1000 nM SS-14. Data is presented as mean±SEM. n = 12 wells (pooled from 2 different experiments). Each treatment group was compared with the control group using Independent t-test. An asterisk (*) represents values that are significantly different from the control.

A. Effect of GHS-Ra on $gGRL_{[19]}$ induced GH release from dispersed pituitary cells



B. Effect of GHS-Ra on $gGRL_{[19]}$ induced LH release from dispersed pituitary cells

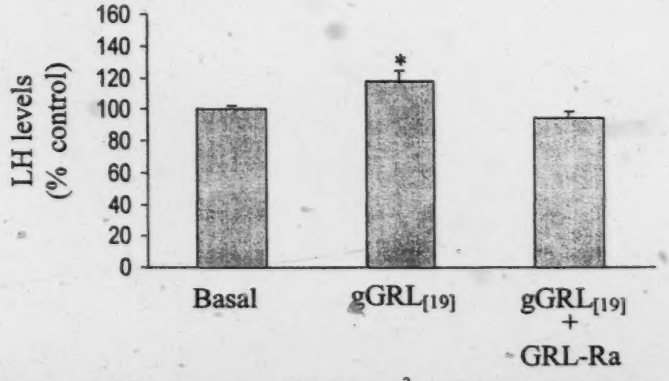
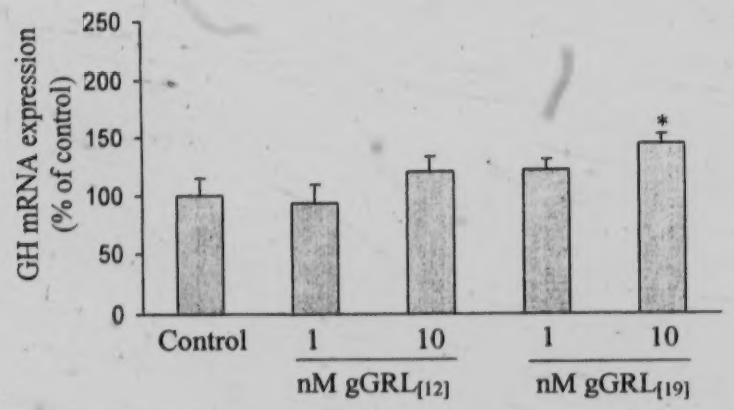


Figure 4.7. Effect of GHS-R antagonist, D-Lys^3-GHRP-6 on $gGRL_{[19]}$ induced GH (A) and LH (B) release from dispersed pituitary cells in static culture. Bars represent the GH or LH levels in control (plain testing media alone), 10 nM $gGRL_{[19]}$ or 10 nM $gGRL_{[19]}$ + 1000 nM D-Lys^3-GHRP-6. Data is presented as mean±SEM. n = 8 wells (pooled from 2 different experiments). Each treatment group was compared with the control group using Independent t-test. An asterisk (*) represents values that are significantly different from the control.

A. Effects of gGRL on GH mRNA expression in goldfish somatotropes



B. Effects of gGRL on LHβ mRNA expression in goldfish gonadotropes

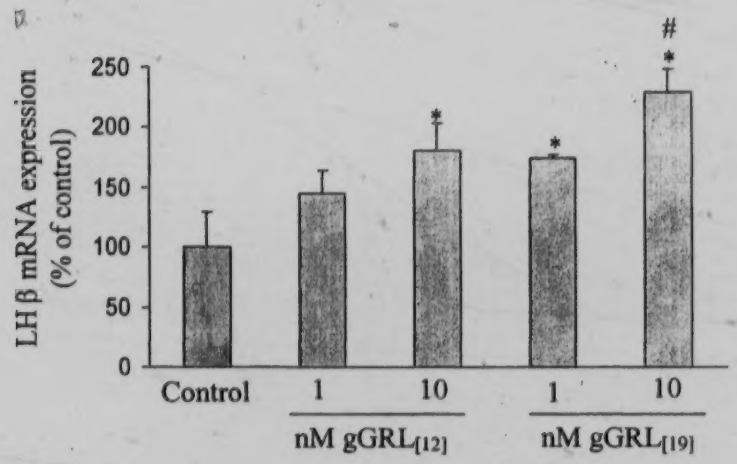
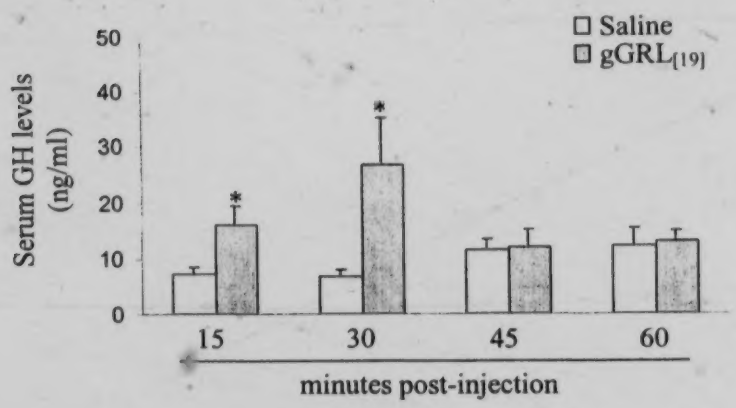


Figure 4.8. Effect of different concentrations of $gGRL_{[12]}$ or $gGRL_{[19]}$ on GH (A) and LHβ (B) mRNA expression in dispersed pituitary cells. Data is presented as mean±SEM. n = 4 wells (from a single experiment). Values were compared by ANOVA followed by least significance difference test. An asterisk (*) represents values that are significantly different from the control. The sign (#) represents the value that is significantly higher than the other treatment groups.

A. Effects of ICV injections of $gGRL_{[19]}$ on serum GH levels



B. Effects of ICV injections of $gGRL_{[19]}$ on serum LH levels

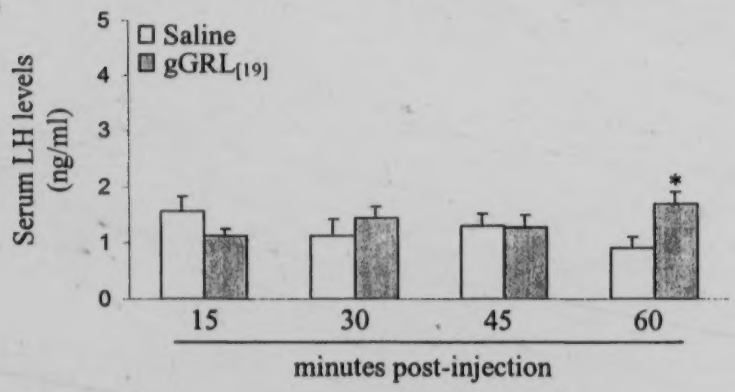
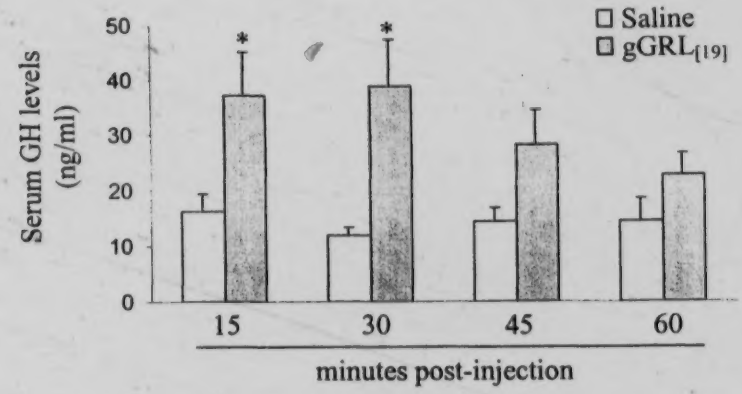


Figure 4.9. Effect of intracerebroventricular (ICV) injections of 1ng/g BW of $gGRL_{[19]}$ or fish physiological saline (control groups) on serum GH (A) and LH (B) levels in goldfish at 15, 30, 45 or 60 minutes post-injection. Data is presented as mean±SEM. n = 6 fish per group. Saline injected group (white bars) for each time point (15, 30, 45 or 60 minutes) was compared to the respective ghrelin injected group (grey bars) using Independent T-test. An asterisk (*) denotes significant difference ($p < 0.05$) compared to the respective saline treated control.

A. Effects of IP injections of $gGRL_{[19]}$ on serum GH levels



B. Effects of IP injections of $gGRL_{[19]}$ on serum LH levels

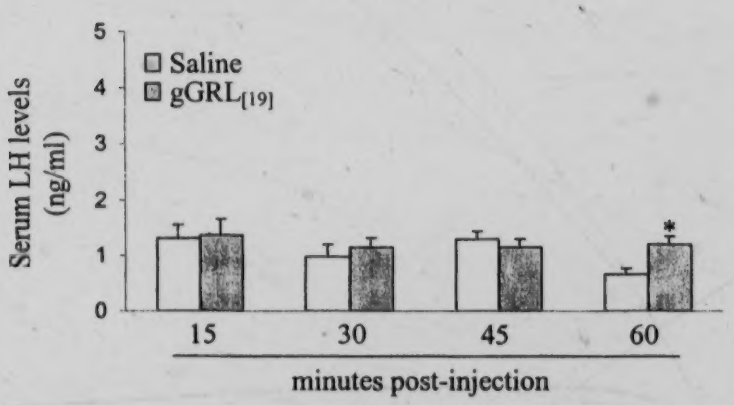


Figure 4.10. Effect of intraperitoneal (IP) injections of 100ng/g $gGRL_{[19]}$ or fish physiological saline (control groups) on serum GH (A) and LH (B) levels in goldfish at 15, 30, 45 and 60 minutes post-injection. Data is presented as mean±SEM. n = 8 fish per group. Saline injected group (white bars) for each time point (15, 30, 45 or 60 minutes) was compared to the respective ghrelin injected group (grey bars) using Independent T-test. An asterisk (*) denotes significant difference ($p < 0.05$) compared to the respective saline treated control.

CHAPTER 5

CHARACTERIZATION OF COMPLEMENTARY DEOXYRIBONUCLEIC ACIDS ENCODING PREPROGALANIN AND ITS ALTERNATIVE SPLICE VARIANTS IN THE GOLDFISH

5.1. Introduction

Galanin is a 29 amino acid neuropeptide, originally isolated from the porcine intestine (Tatemoto et al., 1983). Galanin mature peptide is 29 amino acids in length in all species from which it has been identified, except human galanin, which is a non-amidated 30 amino acid peptide. Galanin peptide has been purified from chicken (Norberg et al., 1991), alligator (Wang and Conlon, 1995) and fish species including the trout (Anglade et al., 1994), tuna fish (Habu et al., 1994), bowfin (Wang and Conlon, 1994), dogfish (Wang and Conlon, 1994) and sturgeon (Wang et al., 1999). Human (Evans and Shine, 1991) and mouse (Kofler et al., 1996) preprogalanin genes are comprised of six exons and five introns, with similar organization. The three main regions in the mammalian preprogalanin mRNA are regions encoding for the signal peptide region, the mature peptide region and the GMAP region (Kofler et al., 1996). Recently, the mRNAs encoding two forms of the preprogalanin have been identified in the quail brain (Kohchi and Tsutsui, 2000). Quail preprogalanin normal

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A version of this chapter has been published. Reprinted from publication: **Unniappan S, Lin X, Peter RE 2003** Characterization of complementary deoxyribonucleic acids encoding preprogalanin and its alternative splice variants in the goldfish. ***Molecular and Cellular Endocrinology*** 200: 177-187; with copyright permission from *Elsevier*.

form has a 29 amino acid galanin mature peptide, while the alternate transcript has an insert of 69 nucleotides in the mature peptide region. The N-terminal 1-14 amino acids (GWTLNSAGYLLGPH) of the galanin mature peptide are conserved in all species from which galanin has been identified, except in the tunafish where a serine (S) at position 6 has been substituted by an alanine (A) (Habu et al., 1994).

In rats and humans, galanin immunoreactivity is present in several regions of the brain (Melander et al., 1986, Merchenthaler et al., 1990) and several peripheral organs including the pituitary, intestine, pancreas, heart, kidney, and human liver (Palkovits et al., 1987, El-Salhy et al., 1993). Galanin like immunoreactivity has been shown in the brain and pituitary of several teleost fish (*see* Jadhao and Pinelli, 2001), including the goldfish (Rao et al., 1996). Sexual dimorphism in the brain distribution of galanin has been shown in several fish species including the molly (Cornbrooks and Parsons, 1991a, 1991b), goldfish (Rao et al., 1996) and salmon (Jadhao and Meyer, 2000). In goldfish, males have a wider galanin immunoreactive fiber system than females (Rao et al., 1996).

There are no reports available on the galanin gene or preprogalanin cDNA structure from any fish species. The objective of the present study is to identify the messenger ribonucleic acids (mRNAs) encoding preprogalanin peptides in goldfish.

5.2. Materials and methods

Animals

Male and female goldfish (*Carassius auratus*) of the common or comet varieties and approximately 35g body weight, were purchased from the Mount

Parnell Fisheries (Mercersberg, PA, USA) and maintained under a simulated natural photoperiod of Edmonton, Alberta, Canada. Fish were maintained in 300 l flow-through aquaria that received a constant flow of aerated and dechlorinated city water at 17^{0}C, and fed daily with commercially prepared Unifeed Nu-Way trout ration (United Feeds, Calgary, Alberta, Canada). Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) before decapitation and dissection of the brain and other tissues. Sex of the fish was determined by examination of the gonads. All protocols were approved by the Department of Biological Sciences Animal Care Committee, University of Alberta.

Reagents and test substances

Trizol reagent, Taq DNA polymerase and the SuperScript II Preamplification system were purchased from the Gibco-Invitrogen Life Technologies (Burlington, Ontario, Canada). Geneclean II Kit was purchased from BIO/CAN Scientific (Mississauga, Ontario, Canada). The oligonucleotides were purchased from the Gibco-Invitrogen Life Technologies or synthesized in the Molecular Biology Service Unit (Department of Biological Sciences, University of Alberta). pGEM-T vector system and the TdT enzyme kit were purchased from Promega (Madison, WI). Hybond nylon membrane, Rediprime-II Quick Prime Kit and [α-^{32}P] deoxy-CTP (dCTP) were obtained from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, England). QIAquick oligonucleotide removal kit was purchased from Qiagen Inc. (Mississauga, Ontario, Canada).

Cloning of the mRNAs encoding galanin mature peptide region using RT-PCR

Total RNA was extracted from the goldfish brain (whole brain tissue from several fishes pooled together) using the Trizol reagent and cDNA was synthesized using the SuperScript II Preamplification System. PCR was performed using two degenerate primers designed based on the cDNA sequences of mammalian galanin (Vrontakis et al., 1987, Kofler et al., 1996). The PCR product was fractionated on a 2% agarose gel and the distinct band of approximately 100 basepair (bp) was excised and the DNA was eluted into 10 M ammonium acetate from the gel using a Model UEA Unidirectional Electroelutor (International Biotechnologies Inc., New Haven, Connecticut). The purified DNA fragment was subcloned into the pGEM-T vector and sequenced with T7 and SP6 primers using a CEQ 2000 automated sequencer (DNA Core Facility, Department of Biochemistry, University of Alberta). Analysis of this partial cDNA sequence and the deduced amino acid sequence showed that it has high similarity with the nucleotide and amino acid sequences of the mammalian galanin.

Rapid amplification of cDNA ends (RACE) and RT-PCR for the identification of the full-length cDNA

To isolate the 3' end of the goldfish preprogalanin cDNA, RT and RACE was conducted as described previously (Lin et al., 1999) using two gene specific primers designed based on the partial cDNA sequence encoding for goldfish galanin mature peptide region. A distinct band of approximately 400 bp was obtained. This band was excised, purified using the GeneClean II kit, subcloned into the pGEM-T vector and sequenced as

described earlier. Two different nucleotide sequences were identified during the analysis. These sequences were named form 1 cDNA and form 2 cDNA. Gene specific primers were designed based on the 3' sequences and 5' RACE was conducted as described earlier (Lin et al., 1999) to obtain the 5' end sequences of the form 1 and form 2 cDNA.

In order to obtain the full-length cDNA sequence of preprogalanin form 1, a forward primer, Gal1-F (5'GGAGGAACAGGAATATTAGC3') and reverse primer Gal1-R (5'GTCGAATGCATTTATAGG3'), were designed based on the 3' and 5' sequences of the form 1 cDNA. RT-PCR was conducted using these primers with denaturation at 95 C for 1 min, followed by 35 cycles of denaturation at 95 C for 1 min, annealing at 53 C for 1 min and extension at 73 C for 1 min. Two bands of approximately 600 bp and 650 bp were obtained. These bands were excised, purified and subcloned into the pGEM-T vector system and sequenced as described earlier.

Gene specific primers, Gal2-F (5'CAGATGAACGGTGTGTCGG3') and Gal2-R (5'CCATCTCCACCTGAGCCT3') based on the 3' and 5' cDNA sequences of form 2, were designed for RT-PCR to derive the full-length cDNA. The conditions for this RT-PCR were the same as that of the RT-PCR for obtaining the full length form 1 cDNA, except for the annealing temperature, which was 59 C. Two bands of approximately 450 bp and 500 bp length were obtained. These two bands were excised, purified and subcloned into the pGEM-T vector system and sequenced as described earlier.

RT-PCR followed by cloning and sequence analysis were repeated for each set of sequences to confirm that the sequences did not include any errors caused by misincorporation during PCR.

PCR for intron trapping

Primers, Gal1-intron3F (5' AAAGAGAAGAGAGAGGATGGAC3') and Gal1-intron3R (5' CCTGCCACTCCGTGCTTGTC3') were used to identify the intron 3 (based on the mammalian preprogalanin gene organization) of the goldfish galanin gene 1. 100 ng genomic DNA was used for the PCR. The PCR conditions were denaturation at 95 C for 5 mins followed by 35 cycles of denaturation at 95 C for 1 min, annealing at 54 C for 1 min and 30 seconds and extension at 73 C for 1 min, and a final extension at 73 C for 15 mins. Introns 4 and 5 (based on the mammalian galanin gene organization) of preprogalanin form 1 were identified using specific primers, Gal1-intron5F (5' CACGGAGTGGCAAGGCAGAG3') and Gal1-intron5R (5'TCTGACGTGAGAGAGGGAAGG3') designed based on the galanin form 1 cDNA. The conditions for this PCR was the same as that explained above except that the annealing, extension and final extension times were 1 min, 2 mins and 10 mins, respectively.

Similarly, PCR was conducted using Gal2-intron3F (5'AGGATGGACC CTCAACAGTG3') and Gal2-intron3R (5'TGTCACTAAGGCAAGAG3') based on the preprogalanin form 2 cDNA to identify the structure of intron 3 in the goldfish galanin gene 2. The PCR conditions were similar to the PCR conditions described for obtaining the intron 3 of galanin gene 1, except that the annealing temperature for this PCR was 56 C. Purification, cloning and sequencing of the desired band were conducted as described earlier.

RT-PCR detection of preprogalanin mRNAs in the brain, pituitary and peripheral tissues of goldfish

Tissues from distinct regions of the goldfish brain, pituitary, intestine, liver, spleen, muscle, gill, heart, kidney, eye, testis, ovary and testis were excised, and total RNA extracted and first strand DNA synthesized as described earlier. RT-PCR was conducted using specific primers used to obtain the complete sequence of preprogalanin 1 and preprogalanin 2. The PCR conditions for preprogalanin form 1A were denaturation at 95 C for 5 mins followed by 30 cycles of denaturation at 95 C for 1 min, annealing at 53 C for 1 min and extension at 73 C for 1 min. For the preprogalanin form 2, the PCR conditions were the same except that the annealing temperature was 59 C. The products of PCR were fractionated on a 1.5% agarose gel and transferred on to Hybond nylon membrane. The membranes were then hybridized with [α-^{32}P] dCTP labeled preprogalanin form 1B or form 2B probe, respectively. Hybridization was performed using methods described by Lin et al. (1999), except for the purification of the labeled probe using the QIAquick oligonucleotide removal kit prior to hybridization following the manufacturer's guidelines. Briefly, after hybridization overnight at 65 C, the membranes were washed two times with washing solution (0.04 M $NaHPO_4$, 1mM EDTA, and 1% SDS) at 65 C. The membranes were then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 1 hour and the screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). As an internal control, RT-PCR

was performed on all cDNAs with the primers for goldfish β-actin (unpublished results, GenBank accession number AF079831). The conditions of this PCR were denaturation at 95 C for 1 min followed by 25 cycles of denaturation at 95 C for 1 min, annealing at 55 C for 1 min and extension at 73 C for 1 min.

Northern blot analysis

Northern blot analysis was carried out to detect the brain, pituitary and the gonadal distribution of the preprogalanin form 1 mRNA. Tissues of five distinct regions of goldfish brain [olfactory bulbs and tracts, telencephalon, hypothalamus, midbrain (optic tectum and thalamus), posterior brain (cerebellum, medulla and spinal cord)] and pituitary gland were collected and total RNA was extracted separately from the male and female goldfish using TRIzol reagent. Approximately 20 μg of total RNA from pooled tissues of male or female goldfish were electrophoresed separately in a denaturing agarose gel (1.5%) with formaldehyde and blotted on to a Hybond nylon membrane by capillary transfer. The probe used for this Northern analysis covers the full-length sequence of preprogalanin form 1A. A trial experiment with a probe covering the preprogalanin form 1A and 1B did not detect two signals representing the two forms, unlike in the RT-PCR analysis. The hybridization was conducted as described earlier. To check the integrity of the RNA used for the Northern blot, the membranes were stripped and reprobed with an [α-^{32}P]dCTP labeled partial cDNA probe for goldfish β-actin.

5.3. Results

Cloning of the mRNAs encoding multiple forms of preprogalanin

Form 1 mRNAs

The 100 bp sequence obtained from RT-PCR showed high nucleotide and amino acid similarity with the mammalian galanin mature peptide sequences. Additionally, the N-terminal 14 amino acid conserved region was present in this sequence. 3' RACE resulted in a 350 bp band. Two different sequences, one longer than the other, were revealed during the sequence analysis of the clones containing an insert obtained from this band. These sequences were designated as form 1 and form 2 respectively. This nomenclature was assigned based on our hypothesis that two different genes encode these two putative forms of galanin.

Sequence analysis of the clone representing the distinct band of approximately 450 bp obtained during the 5' RACE for preprogalanin form 1 revealed a 362 bp sequence. Comparison of this sequence with the nucleotide sequence obtained after the 3' RACE sequence showed the presence of overlapping regions. 72 nucleotides were missing from the GMAP region in the 5' RACE sequence compared to the 3' RACE sequence.

RT-PCR conducted on the whole brain cDNA using specific primers based on the 3' and 5' cDNA ends of form 1 produced two bands of approximately 650 bp and 600 bp. Sequence analysis of the clone representing the 600 bp band represents the normal preprogalanin sequence of 566 bp (GenBank Accession number, AF 416924). This 118 amino acid preprogalanin sequence consists of a signal peptide,

mature galanin peptide and the GMAP. The 31 amino acid signal peptide was identified, which ends in a proteolytic processing site, lysine at position 30 and arginine at position 31. The 29 amino acid mature peptide region follows the signal peptide and end with the dibasic cleavage site and the amidation signal (GKR). A 54 amino acid GMAP region follows the mature peptide and ends in the stop codon. This sequence has been designated as preprogalanin form 1 A (Figure 5.1).

The analysis of the second sequence (obtained from the 650 bp band) revealed that it has an additional 72 nucleotides (24 amino acids) insert in the middle of the region encoding the mature peptide. This sequence is 634 bp long compared to the preprogalanin form 1A sequence (GenBank Accession number, AF416925). The deduced amino acid sequence of the preprogalanin form 1A nucleotide sequence is 142 amino acids long. The insert begins after the first 13 amino acids of the N-terminal conserved region (GWTLNSAGYLLGP; Figure 5.1). All other regions of this sequence are identical to the preprogalanin form 1A, except for the change of an amino acid histidine (H) to tyrosine (Y) at the 14 position of the N-terminal region of the mature peptide. This sequence has been named preprogalanin form 1B.

Preprogalanin form 1A and preprogalanin form 1B sequences were compared with the 5' RACE sequence for galanin form 1. Sequence comparison revealed that the 362 bp sequence (GenBank Accession number, AF416926) from the 5' RACE sequence is identical to the preprogalanin form 1A (normal form) except for the deletion of 72 nucleotides (23 amino acids; splicing cleaves one triplet codon, which leads to three nucleotides coding no amino acids) from the GMAP region (Figure

5.1). No insert in the mature peptide region, as seen in the preprogalanin 1B, has been observed. This sequence, which is 95 amino acids long has been designated as preprogalanin 1C.

Form 2 mRNAs

Two bands of 400 bp and 450 bp were obtained from the RT-PCR to determine the full-length sequence of form 2 cDNA. Sequence analysis of the clone representing the 400 bp band revealed a 398 bp sequence (GenBank Accession Number, AF416928). This encodes a 119 amino acid preprogalanin with a 32 amino acid signal peptide region, 31 amino acid mature peptide region followed by a dibasic cleavage site and an amidation signal (GKR) and a 53 amino acid GMAP region. This nucleotide sequence has been designated as preprogalanin form 2A (Figure 5.2) encoded by galanin gene 2. The second band of approximately 450 bp represents a sequence of 471 bp (GenBank Accession number, AF415929). This sequence (143 amino acids long) has an insert of 72 nucleotides (24 amino acids; Figure 5.2) in the mature peptide region compared to the preprogalanin 2A. The fourteenth amino acid has been changed from histidine (H) to tyrosine (Y) due to the insert. This sequence has been designated as the preprogalanin 2B cDNA.

PCR for galanin intron trapping

For gene 1, the distinct band of 1700 bp, obtained after the PCR on the genomic DNA, was sequenced. This sequence (GenBank Accession number, AF 416927; sequence not shown) has the intron 3 of the galanin gene and parts of the exons (exon 3 and 4) on which the specific primers were designed. The 600 bp band

obtained after the genomic DNA PCR for identifying the introns in the GMAP region, represents a 648 bp sequence (GenBank Accession number, AF416927; sequence not shown). These are the complete sequences of introns 4 (204 bp) and 5 (223 bp), and the complete sequence of exon 5 (24 amino acids) and the partial sequences of exons 4 and 6.

Similarly, the genomic DNA PCR for identifying intron 3 of the galanin gene 2 resulted in a distinct band of 1500 bp. The sequence of this 1500 bp PCR product (GenBank Accession number, AF416931; sequence not shown) represents the full sequence of intron 3 and the partial sequences of exons 3 and 4. All intron:exon junctions in goldfish galanin genes conforms with the "GT-AG" rule of introns (Breathnach and Chambon, 1981).

Tissue specific expression of galanin mRNA

The distribution of the preprogalanin form 1A and its splice variants, and the preprogalanin form 2B and its splice variant were examined using RT-PCR followed by Southern blot analysis. Two strong bands representing preprogalanin form 1A and preprogalanin form 1B were found in the telencephalon and hypothalamus, a single band representing preprogalanin 1B was found in the eye and preprogalanin 1A was found in the ovary (Figure 5.3.A, 5.3.B). Presence of preprogalanin 2A and 2B were detected in the telencephalon, hypothalamus, mid brain (optic tectum-thalamus), and the posterior brain. Preprogalanin form 2A was also detected in the intestine, liver, spleen, and 2B in the testis and ovary (Figure 5.4.A, 5.4.B). The preprogalanin form 1C was undetectable in any of the tissues; RT-PCRs using

cDNAs from various tissues, specific primers for preprogalanin form 1C and different PCR conditions to detect the preprogalanin 1C were all unsuccessful. Control PCRs with primers without the template did not obtain any bands. Electrophoresis of the β-actin PCR products showed a band of 580 bp in all lanes, demonstrating that the cDNAs from various tissues used were of good quality (Figure 5.3.C, 5.4.C).

In the Northern blot analysis, a signal for preprogalanin 1A mRNA was detected in the telencephalon, hypothalamus and midbrain of both male (Figure 5.5.A, 5.5.B) and female goldfish (Figure 5.5.C, 5.5.D). Additionally, expression was identified in the olfactory bulbs and tracts of female goldfish. A weak signal was detected in the posterior brain of the female goldfish. No expression was found in the testis, while a faint signal was detected in the ovary.

5.4. Discussion

Preprogalanin cDNAs

In this study, multiple forms of mRNAs encoding preprogalanin form 1 and preprogalanin form 2 in the goldfish were identified. Comparison of the goldfish preprogalanin amino acid sequences with other known preprogalanin sequences is shown in Figure 5.6. Within the different forms of preprogalanin mRNAs in the goldfish, the highest sequence identity is between the preprogalanin 1B and 2B (91%) followed by preprogalanin 1A and preprogalanin 2A (90%). Goldfish preprogalanin sequences have 46-58% sequence identity to the quail preprogalanin

sequences. All forms of goldfish preprogalanin sequences have the highest identity (40-50%) with the rat preprogalanin sequence among the mammalian sequences.

The deduced amino acid sequences suggest that the mature peptide region of preprogalanin form 1A and 1C could produce an amidated mature peptide of 29 amino acids. The mature peptide formed from the preprogalanin 2A precursor peptide is 31 amino acids. There are two extra amino acids (a serine and a leucine at positions 24 and 25, respectively) present in this mature peptide, compared to the mature peptide formed from the preprogalanin 1A, which has 29 amino acids. Here we present evidence for a galanin mature peptide derived from the goldfish preprogalanin form 2 A that is longer than the human galanin. The longest galanin form known so far is the human galanin with 30 amino acids (Evans et al., 1991). Notably, no differences in the physiological functions of human galanin compared to the other known galanin peptides have been reported. The reason for the similar functions may be due to the conserved N-terminal region. In goldfish, the 31 amino acid galanin peptide formed from the preprogalanin 2A has a conserved N-terminal 1-14 region and may also have similar physiological functions as that of the other galanin peptides.

Even though there is a 72 nucleotides insert in the preprogalanin form 1B and 2B, we do not know whether this region would be retained during the translation and posttranslational processing of the preprogalanin precursor to produce the galanin mature peptide. The insertion, however, is not disrupting the open reading frame, which suggests that two forms of preprogalanin peptides may be produced.

The insert in the mature peptide region of the quail preprogalanin long transcript is 23 amino acids (69 nucleotides) (Kohchi and Tsutsui, 2000), while the insert in the mature peptide region of the goldfish preprogalanin 1B and 2B is 24 amino acids (72 nucleotides). The long transcript of quail preprogalanin and the goldfish preprogalanins 1B and 2B have the insert starting after the 13th amino acid (proline) position in the conserved N-terminal region of the mature peptide. The first six amino acids (RRIDHL) in the inserted region of the quail preprogalanin are identical in the goldfish preprogalanin forms 1B and 2B.

This is the first report of a preprogalanin sequence (preprogalanin 1C) with a deletion of 23 amino acids in the GMAP region. Functional significance of the GMAP region is not well characterized. However, Xu et al., (1995) reported that the GMAP has an effect on the flexor reflex in rats, suggesting some potential physiological roles.

Two genes encode GH (Chan et al., 1996), salmon and chicken GnRH (Lin and Peter, 1997) and prolactin (Law et al., 1996) in the goldfish. Recently, two mRNAs encoding the cocaine and amphetamine regulated transcript (CART) were identified in the goldfish (Volkoff and Peter, 2001). We assume that the preprogalanin form 1 mRNAs are derived from the galanin gene 1 and the preprogalanin form 2 mRNAs originated from the galanin gene 2. Goldfish are tetraploid. Risinger and Larhammar (1993) first demonstrated the duplicate loci and sequence diversity in the goldfish for the synapse protein, SNAP-25, consistent with the tetraploidization event that has occurred 15-20 million years ago. We are not

sure whether the presence of duplicate galanin genes has occurred due to an earlier gene duplication event or the tetraploidization event of 15-20 million years ago.

Multiple forms of preprogalanin mRNAs - Are they splice variants?

We hypothesized that the three forms of preprogalanin mRNA 1 (1A, 1B and 1C) and two forms of preprogalanin mRNA 2 (2A and 2B) are splice variants that arise from the duplicate genes, galanin gene 1 and gene 2, respectively (Figure 5.7). The 72 nucleotide inserts in the preprogalanin 1B and 2B start with "GT" and ends in "AT". The quail preprogalanin long form also has been identified as an alternative splice variant, with the splice junctions starting in "GT" and ending in "AT" (Kohchi and Tsutsui, 2000).

If the intron:exon organization of the goldfish galanin gene is similar to that of the mouse (Kofler et al., 1996), the insertion or deletion of nucleotides occurring in the goldfish preprogalanin forms are likely located within the intron sequences of the goldfish galanin genes (Figure 5.7). In the mouse the location of intron 3 is in between the exons (exon 3 and exon 4) coding for the mature peptide. We hypothesize that the 72 nucleotides inserted in the preprogalanin 1B and 2B mRNAs encoding for the mature peptide region are a part of the intron 3. The results of the intron trapping provide supportive evidence for this proposal. The 72 nucleotides that are inserted into the preprogalanin form 1B and form 2B are present in the intron 3 sequence of galanin gene 1 and gene 2. Breathnach and Chambon (1981) suggested a stepwise mechanism, dependent on the availability of splicing factors and enzymes, by which large introns can be removed. The retention of a part (72

nucleotides) of the intron 3 in preprogalanin form 1B and preprogalanin form 2B may have resulted from the premature termination of the splicing of intron 3, due to the absence of necessary splicing factors or enzymes (Figure 5.8).

Similarly, we hypothesize that the deletion of 72 nucleotides from the GMAP region in the preprogalanin 1C mRNA is due to alternate splicing. The results of the intron trapping support this hypothesis. The 72 nucleotides (24 amino acids) deleted from the preprogalanin 1C mRNA is from exon 5 in galanin gene 1. There are two short introns, intron 4 (204 bp) and intron 5 (228 bp), located on both sides of the exon 4 (72 nucleotides/24 amino acids in length) that are missing in the preprogalanin form 1C mRNA. In normal splicing, as in the case of preprogalanin 1A, the two short introns (intron 4 and intron 5) will be spliced off and the exon 5 will be retained. However, in preprogalanin form 1C, what may be happening is the splicing off of exon 4 with introns 4 and 5 (Figure 5.8) leading to the absence of the 24 amino acids encoded in exon 5.

Tissue specific expression

Using RT-PCR and Southern blot analysis, expression of preprogalanin form 1A and 1B mRNA have been detected in the telencephalon and the hypothalamus. Additionally, expression of form 1A mRNA in the ovary and form 1B mRNA in the eye of goldfish have been detected. Our inability to detect preprogalanin 1C during the RT-PCR from different brain regions and peripheral tissues suggests that this transcript may be only rarely expressed. Another reason may be the seasonal, sexual or inter-individual variation in the expression of preprogalanin 1C mRNA. Strong

expression of preprogalanin 2A and 2B mRNAs has been detected in the telencephalon, hypothalamus, mid brain, and the posterior brain of the goldfish. Preprogalanin 2A mRNA has been detected in the intestine, liver, and spleen of the goldfish. Preprogalanin 2B mRNA has been detected in the muscle, testis and ovary of the goldfish.

Northern blot analysis indicates a wider distribution of preprogalanin 1A mRNA in the female goldfish. In addition to all regions where galanin is found in the male goldfish, there is a strong expression of preprogalanin in the olfactory bulbs and tracts of the female fish. Extensive galanin immunoreactivity has been demonstrated in several regions of the brain and the pituitary of the goldfish (Rao et al., 1996). The results from the current study confirm the earlier findings from our lab (Rao et al., 1996) indicating that there is a wide distribution of galanin in the goldfish brain. Presence of multiple forms of mRNAs resulting from gene duplication or alternate splicing may be the reason for the species-specific variations in galanin distribution in teleost fish. In goldfish, galanin has been shown to be involved in stimulating food intake and feeding behavior (Volkoff and Peter, 2001). A stimulatory role of galanin in the regulation of GH secretion in fish has been demonstrated (Diez et al., 1991). These results, showing wide distribution of preprogalanin mRNAs in the goldfish, together with the physiological functions reported (Diez et al., 1991, de Pedro et al., 1995, Volkoff and Peter, 2001), suggest that galanin is an important neuropeptide in the fishes.

Gene duplication (Nawa et al., 1984) and alternative splicing (Lou and Gagel, 2000) are natural tools to increase the diversity of peptides. This study present evidence for five novel, putative galanin peptides in the goldfish, encoded by five different mRNAs resulting from alternate splicing of the products from two genes. The significance of these five peptides is unknown. However, the N-terminal 1-14 amino acids, which are critical for receptor binding and activation (Iismaa and Shine, 1999), are fully conserved (compared to other known galanin mature peptides) in the deduced amino acid sequence of preprogalanin 1A, 1C and 2A. The N-terminal 1-13 amino acids are fully conserved in preprogalanin 1B and 2B (compared to preprogalanin 1A, 1C and 2A and other known galanin peptides). This structural diversity suggests that the galanin peptides derived from the multiple preprogalanin mRNAs will enhance the diversity of peptides available for receptor binding, thereby "fine tuning" the physiological functions in which the galanin peptide is involved. More studies are necessary to unravel the mechanisms involved in the alternate splicing of the preprogalanin mRNAs, differential distribution of these mRNAs, structural characteristics of these novel galanin peptides and their physiological functions.

Summary

This study for the first time reports the mRNAs encoding preprogalanin from a fish species. There are at least two galanin genes in the goldfish. Our results suggest that the multiple forms of preprogalanin mRNAs are alternative splice variants. The intron:exon organization of the goldfish galanin genes are similar to

the mammalian galanin gene. Wide distribution of preprogalanin mRNAs was identified in the brain, pituitary and several peripheral organs of the goldfish.

5.5. REFERENCES

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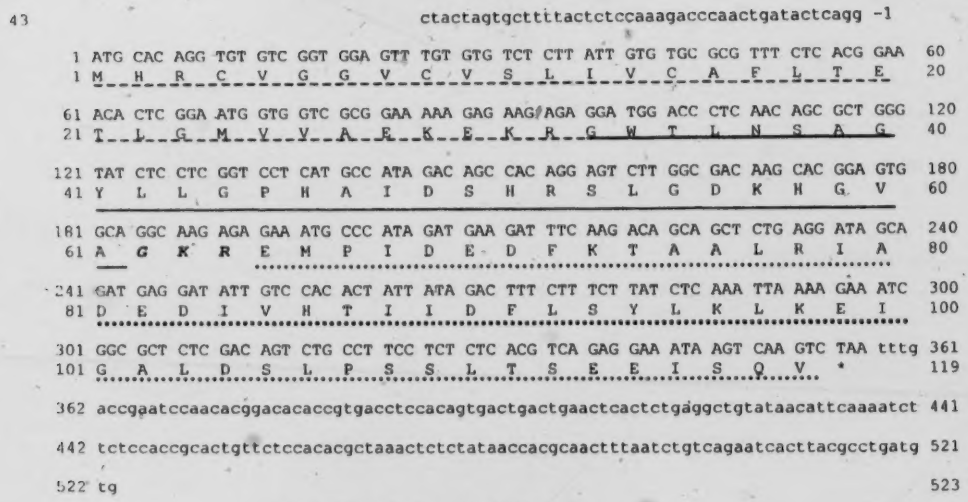
PREPROGALANIN I A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121 TAT CTC CTC GGT CCT CAT GCC ATA GAC AGC CAC AGG AGT CTT GGC GAC AAG CAC GGA GTG 180
41 Y L L G P H A I D S H R S L G D K H G V 60
181 GCA GGC AAG AGA GAA ATG CCC ATA GAT GAA GAT TTC AAG ACA GCA GCT CTG AGG ATA GCA 240
61 A G K R E M P I D E D F K T A A L R I A 80
241 GAT GAG GAT ATT GTC CAC ACT ATT ATA GAC TTT CTT TCT TAT CTC AAA TTA AAA GAA ATC 300
81 D E D I V H T I I D F L S Y L K L K E I 100
301 GGC GCT CTC GAC AGT CTG CCT TCC TCT CTC ACG TCA GAG GAA ATA AGT CAA GTC TAA tttg 361
101 G A L D S L P S S L T S E E I S Q V * 119
362 accgaatccaacacggacacaccgtgacctccacagtgactgactgaactcactctgaggctgtataacattcaaaatct 441
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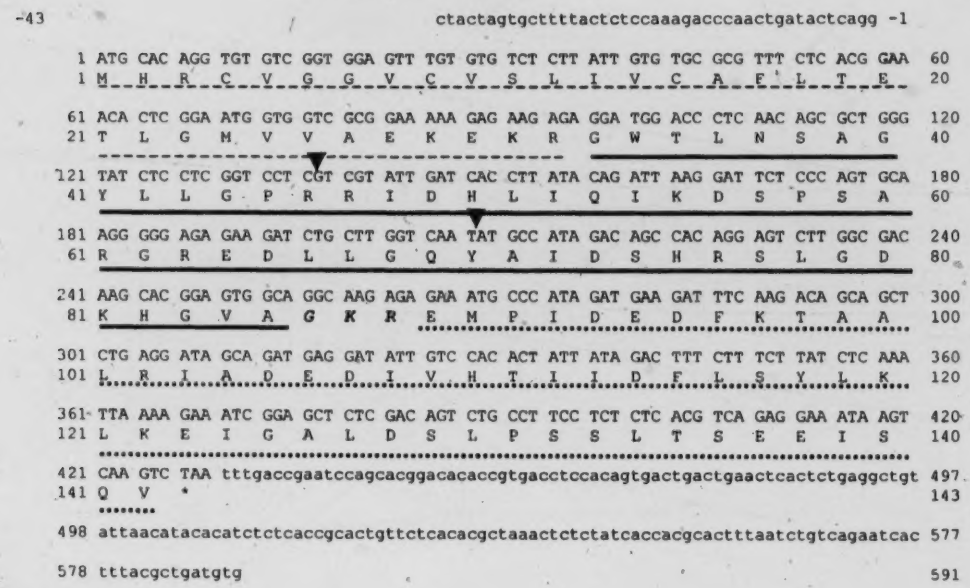
522 tg 523

PREPROGALANIN I B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181 AGG GGG AGA GAA GAT CTG CTT GGT CAA TAT GCC ATA GAC AGC CAC AGG AGT CTT GGC GAC 240
61 R G R E D L L G Q Y A I D S H R S L G D 80
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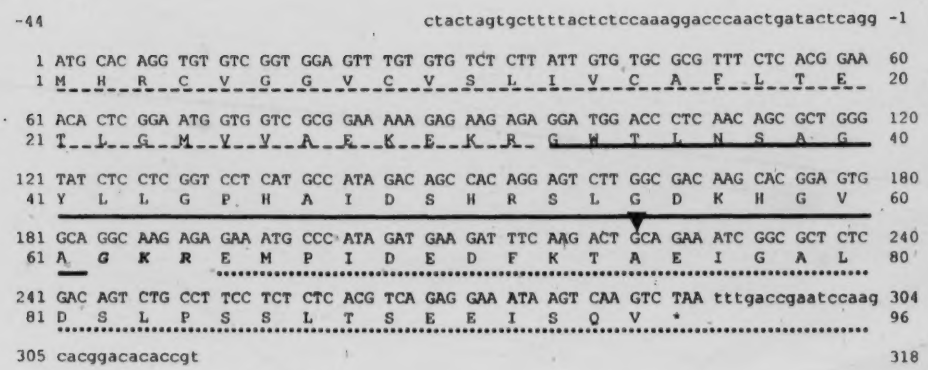
101 L R I A D E D I V H T I I D F L S Y L K 120
361 TTA AAA GAA ATC GGA GCT CTC GAC AGT CTG CCT TCC TCT CTC ACG TCA GAG GAA ATA AGT 420
121 L K E I G A L D S L P S S L T S E E I S 140
421 CAA GTC TAA tttgaccgaatccagcacggacacaccgtgacctccacagtgactgactgaactcactctgaggctgt 497
141 Q V * 143
498 attaacatacacatctctcaccgcactgttctcacacgctaaactctctatcaccacgcactttaatctgtcagaatcac 577
578 tttacgctgatgtg 591

Figure 5.1. Nucleotide sequences of the goldfish preprogalanin form 1 A and B cDNAs. The signal peptide region is underlined with small, broken lines. The mature peptide region is underlined using thick, continuous lines. The 72 nucleotides insertion in the mature peptide region of preprogalanin 1B is shown in a box with the arrow leading to the region of insertion (after "C" of the "CAT" codon for histidine). The amidation signal is italicized. The portion following the amidation signal up to the stop codon is the GMAP region.

PREPROGALANIN I C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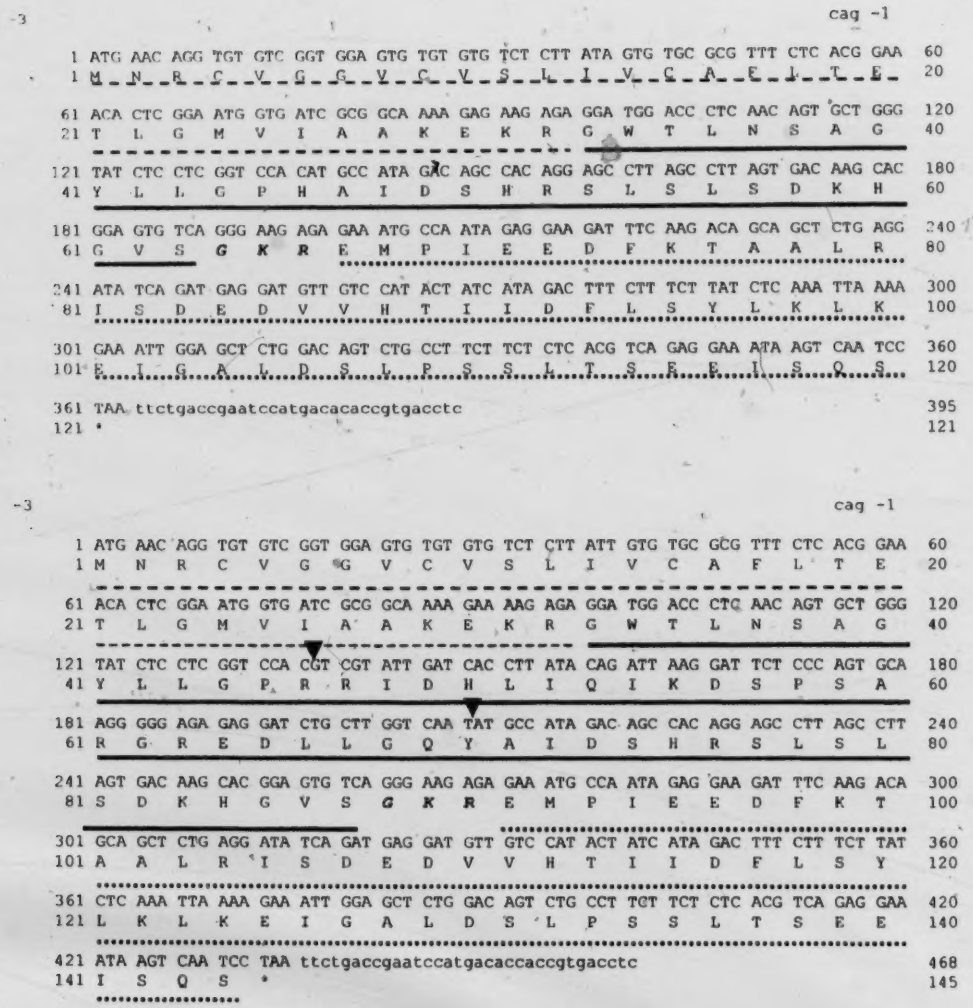
21 T L G M V V A E K E K R G W T L N S A G 40
121 TAT CTC CTC GGT CCT CAT GCC ATA GAC AGC CAC AGG AGT CTT GGC GAC AAG CAC GGA GTG 180
41 Y L L G P H A I D S H R S L G D K H G V 60
181 GCA GGC AAG AGA GAA ATG CCC ATA GAT GAA GAT TTC AAG ACT GCA GAA ATC GGC GCT CTC 240
61 A G K R E M P I D E D F K T A E I G A L 80
241 GAC AGT CTG CCT TCC TCT CTC ACG TCA GAG GAA ATA AGT CAA GTC TAA tttgaccgaatccaag 304
81 D S L P S S L T S E E I S Q V * 96
305 cacggacacaccgt 318

Figure 5.1. Nucleotide sequences of the goldfish preprogalanin form 1C cDNA. The signal peptide region is underlined with small, broken lines. The mature peptide region is underlined using thick, continuous lines. The amidation signal is italicized. The portion following the amidation signal up to the stop codon is the GMAP region. The position of the deletion of 23 amino acids in the GMAP region in the preprogalanin form 1C is indicated by the arrowhead.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121 TAT CTC CTC GGT CCA CAT GCC ATA GAC AGC CAC AGG AGC CTT AGC CTT AGT GAC AAG CAC 180
41 Y L L G P H A I D S H R S L S L S D K H 60
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181 AGG GGG AGA GAG GAT CTG CTT GGT CAA TAT GCC ATA GAC AGC CAC AGG AGC CTT AGC CTT 240
61 R G R E D L L G Q Y A I D S H R S L S L 80
241 AGT GAC AAG CAC GGA GTG TCA GGG AAG AGA GAA ATG CCA ATA GAG GAA GAT TTC AAG ACA 300
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141 I S Q S * 145

Figure 5.2. Nucleotide sequences of the goldfish preprogalanin form 2 A cDNA (top) and 2 B cDNA (bottom). The signal peptide region is underlined with small, broken lines. The mature peptide region is underlined using thick, continuous lines. The 72 nucleotides insertion in the mature peptide region of preprogalanin 2B is shown in a box with the arrow leading to the region of insertion (after "C" of the "CAT" codon for histidine). The amidation signal is italicized. The portion following the amidation signal up to the stop codon is the GMAP region.

Figure 5.3. (A) Gel showing the RT-PCR detection of preprogalanin 1A and 1B in the brain and peripheral tissues of goldfish. The different lanes represent the following; 1 and 18 are molecular weight markers; 2, olfactory bulbs and tract; 3 telencephalon; 4, hypothalamus; 5, mid brain; 6, posterior brain; 7, pituitary; 8, intestine; 9, liver; 10, spleen; 11, muscle; 12, gill; 13, heart; 14, kidney; 15, eye; 16, testis; and, 17, ovary. The band of approximately 600 bp represents preprogalanin 1A and the band of approximately 650 bp represents preprogalanin 1B. (B) The same gel after Southern blot analysis using a ^{32}P-labeled probe covering the entire preprogalanin 1B. (C) Gel showing the β-actin PCR on the same tissues in the same order.

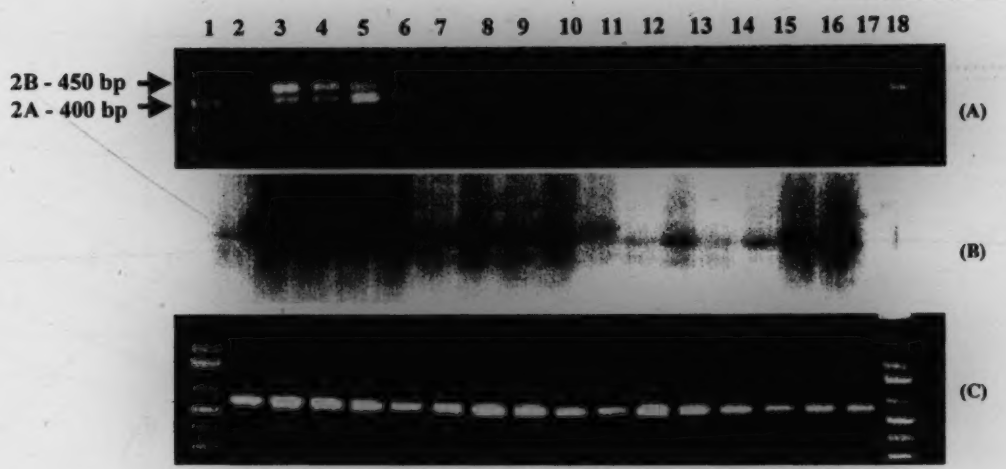

Figure 5.4. (A) Gel showing the distribution of preprogalanin 2A and 2B in the brain and peripheral tissues of goldfish by RT-PCR. The different lanes represent the following; 1 and 18 are molecular weight markers; 2, olfactory bulbs and tract; 3, telencephalon; 4, hypothalamus; 5, mid brain; 6, posterior brain; 7, pituitary; 8, intestine; 9, liver; 10, spleen; 11, muscle; 12, gill; 13, heart; 14, kidney; 15, eye; 16, testis and 17, ovary. The band of approximately 550 bp represents preprogalanin 2A and the band of approximately 500 bp represents preprogalanin 2B. **(B)** The same gel after Southern blot analysis using a ^{32}P-labeled probe covering the entire preprogalanin 2B. **(C)** The picture of the gel showing the β-actin PCR on the same tissues in the same order.

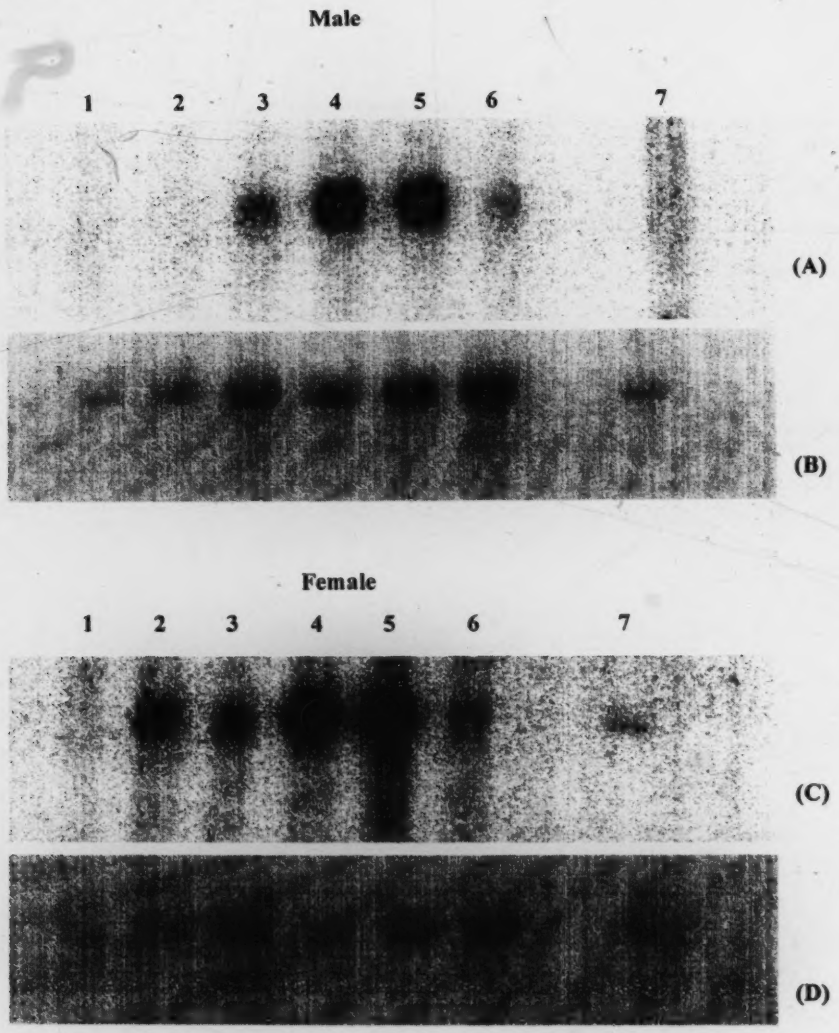

Figure 5.5. (A) Northern blot analysis showing the expression of preprogalanin 1A mRNA in the male goldfish. The different lanes represent the following: 1, pituitary; 2, olfactory bulbs and tracts; 3, telencephalon; 4, hypothalamus; 5, midbrain; 6, posterior brain and 7, testis. **(B)** The membrane (Figure 5.5.A.) was stripped and reprobed using a ^{32}P- labeled β-actin probe. **(C)** Northern blot analysis showing the expression of preprogalanin 1A mRNA in the female goldfish. The different lanes represent the following: 1, pituitary; 2, olfactory bulbs and tracts; 3, telencephalon; 4, hypothalamus; 5, midbrain; 6, posterior brain; and, 7, ovary. **(D)** The membrane (Figure 5.5.C.) was stripped and reprobed using a ^{32}P- labeled β-actin probe.

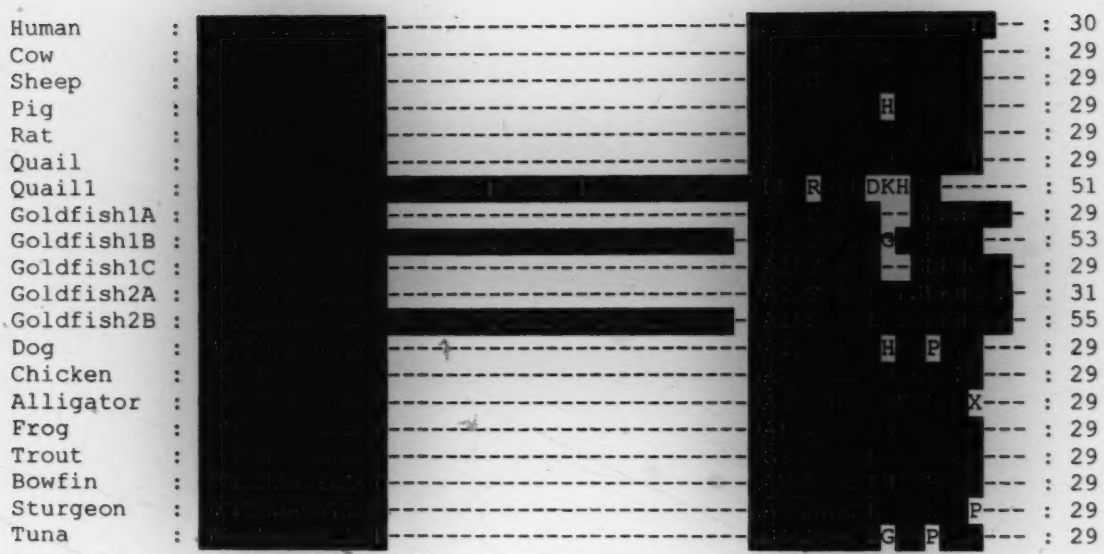
Human : : 30
Cow : : 29
Sheep : : 29
Pig : : 29
Rat : : 29
Quail : : 29
Quail1 : : 51
Goldfish1A : : 29
Goldfish1B : : 53
Goldfish1C : : 29
Goldfish2A : : 31
Goldfish2B : : 55
Dog : : 29
Chicken : : 29
Alligator : : 29
Frog : : 29
Trout : : 29
Bowfin : : 29
Sturgeon : : 29
Tuna : : 29

Figure 5.6. Multiple sequence comparison of galanin peptides from various mammalian and non-mammalian species.

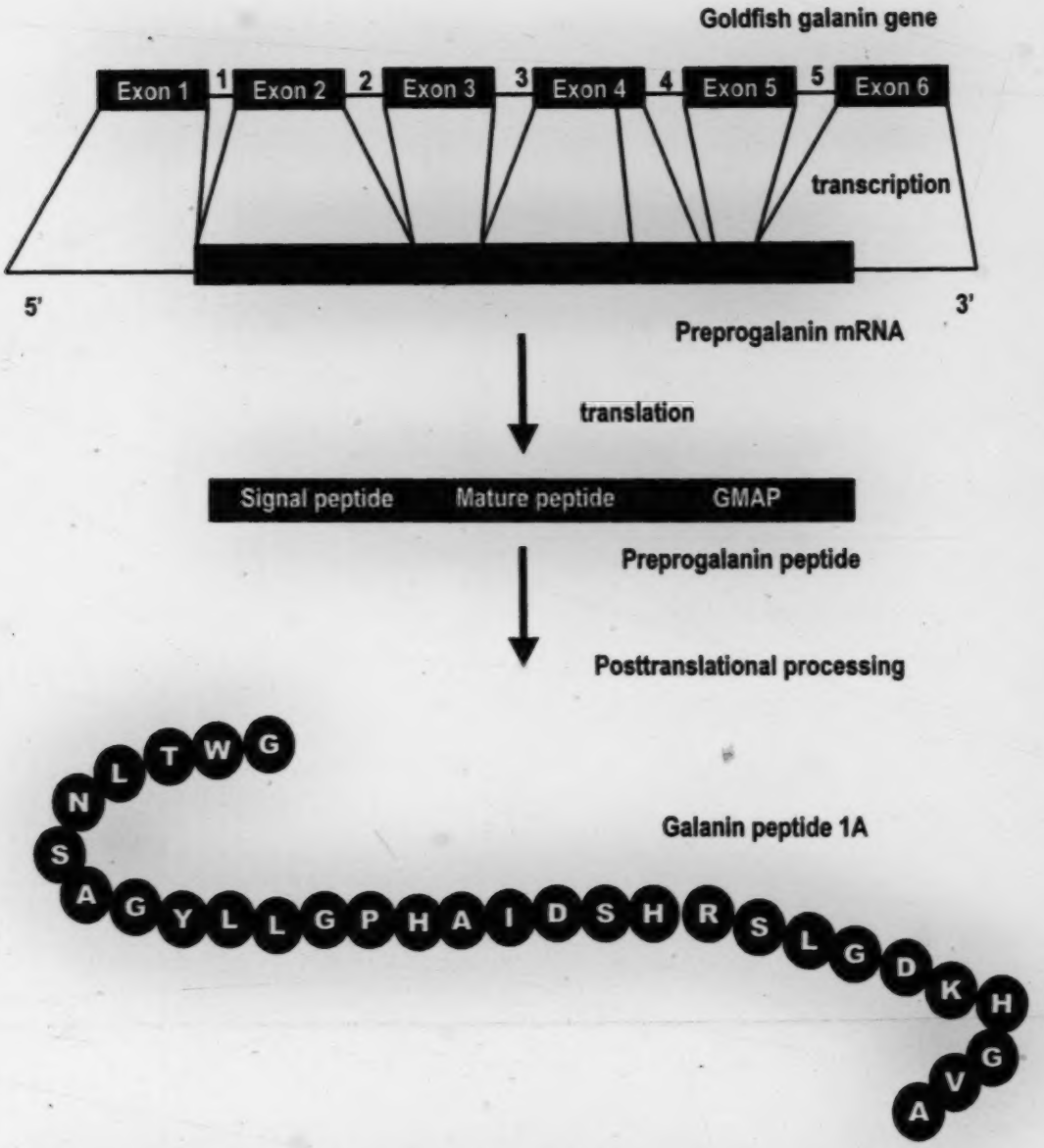

Figure 5.7. Scheme of formation of mature galanin peptide 1A from the galanin gene. Small lines connecting the six exons represents the introns (5 numbers). "GMAP" denotes the galanin message associated peptide region in the preprogalanin peptide. Normal splicing of the introns will result in the preprogalanin 1A mRNA (from gene 1) or 2A mRNA (from gene 2) with a normal signal peptide region, mature peptide region and a GMAP region.

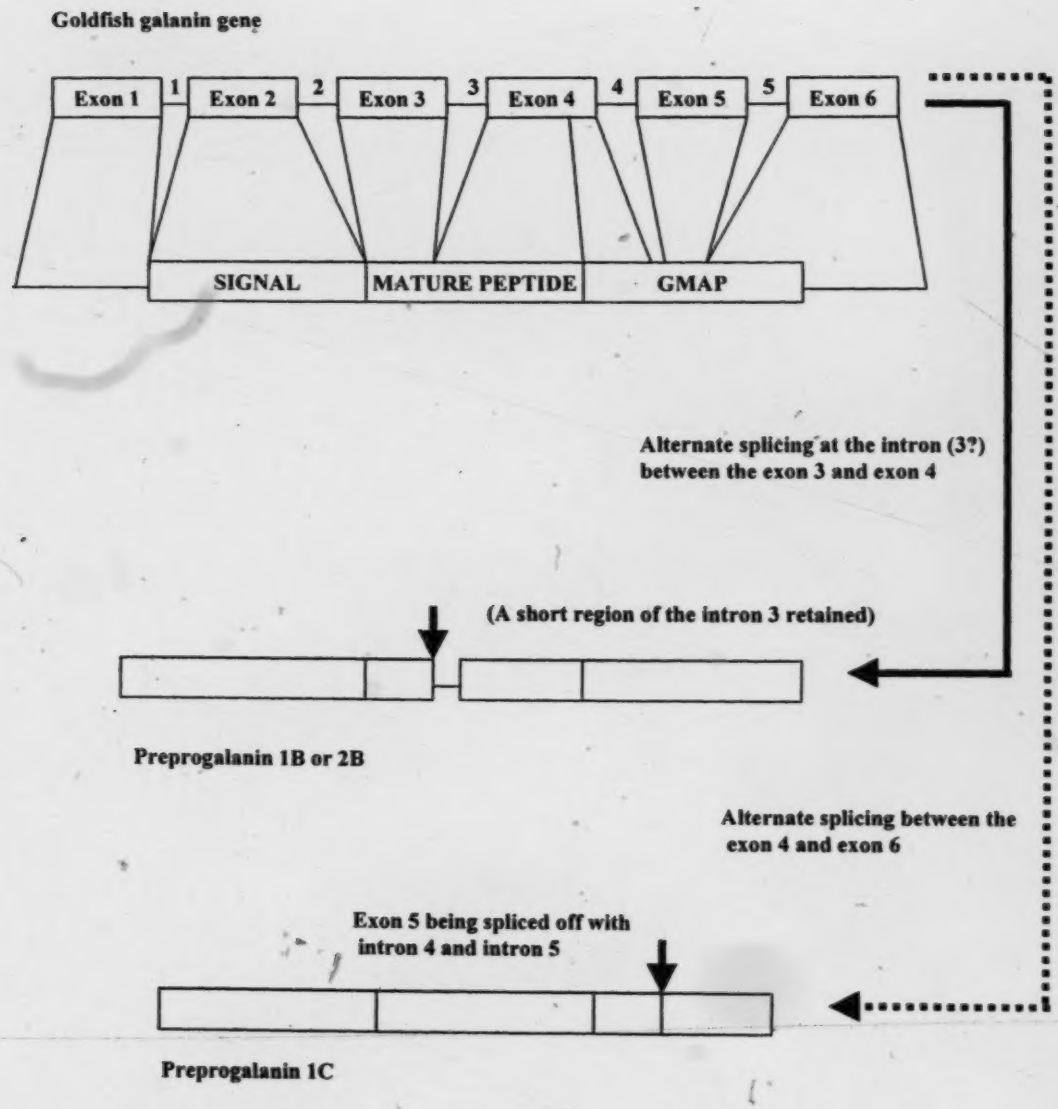

Figure 5.8. Schematic representation of the formation of normal preprogalanin mRNA and its splice variants from the galanin gene. Alternate splicing which results in the retention of a part of the intron 3 will give rise to preprogalanin 1B (from gene 1) or 2B (from gene 2). Exon 5 can be spliced off with intron 4 and intron 5 resulting in preprogalanin 1C, with a deletion in the GMAP region.

CHAPTER 6

IN SITU LOCALIZATION OF PREPROGALANIN mRNA IN THE GOLDFISH BRAIN AND CHANGES IN ITS EXPRESSION DURING FEEDING AND STARVATION

6.1. Introduction

Galanin is a 29 amino acid neuropeptide, originally isolated from the porcine intestine (Tatemoto et al., 1983). Structure of the galanin gene (Corness et al., 1997; Evans and Shine, 1991; Kofler et al., 1995; Kofler et al., 1996; Lundqvist et al., 1995) and peptide (as reviewed in Iisma and Shine, 1999) has been identified in numerous species. Galanin mRNA (Ryan and Gundlach, 1996) and peptide (Merchenthaler et al., 1993) are widely distributed in the brain of mammalian vertebrates. Galanin is involved in the regulation of many physiological functions in mammals (Gundlach, 2002), including the stimulation of food intake (Sahu, 1998; Dube et al., 2000), growth hormone secretion (GH) (Todd et al., 2000) and luteinizing hormone (LH) secretion (Finn et al., 2000). In rats, injections of galanin into the hypothalamus stimulate feeding behavior (Crawley et al., 1990). Central injections of leptin decrease hypothalamic galanin in ad-libitum fed rats (Sahu, 1998). These results indicate that galanin acts in the brain to regulate food intake in mammals.

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Among non-mammalian vertebrates, the structure of galanin has been identified in the quail (Kohchi and Tsutsui, 2000), alligator (Wang and Conlon, 1994a) and many fish species including the trout (Anglade et al., 1994), bowfin (Wang and Conlon, 1994b), dogfish (Wang and Conlon, 1994b), tuna (Habu et al., 1994) and sturgeon (Wang et al., 1999). In teleost fish, a wide distribution of galanin immunoreactivity has been detected in the brain and pituitary of several species (Jadhao and Meyer, 2000; Jadhao and Pinelli, 2001), including the goldfish (Rao et al., 1996). *In vivo* administration of galanin stimulates GH secretion in trout (Diez et al., 1994). Intracerebroventricular (ICV) administration of galanin stimulates food intake in goldfish (de Pedro et al., 1995; Guijarro et al., 1999; Volkoff and Peter, 2001). Guijarro et al. (1999) found that ICV injection of galanin stimulates food intake in the tench (*Tinca tinca*) a cyprinid fish. IP administration of galanin had no effect on food intake in goldfish (de Pedro et al., 1995; Guijarro et al., 1999; Volkoff and Peter, 2001) and tench (Guijarro et al., 1999).

Recently, two genes encoding galanin in the goldfish were identified (Chapter 6). Three alternative splice variant mRNAs are encoded in goldfish galanin gene I, and two splice variants are encoded in galanin gene II (Chapter 6). Northern blot analysis detected preprogalanin mRNA expression in the olfactory bulbs and tracts, telencephalon, hypothalamus, midbrain and the posterior brain of goldfish (Chapter 6). Although galanin is an orexigen, there are no reports available on the preprandial/ postprandial and starvation induced changes in galanin mRNA expression in any non-mammalian vertebrate species. This objectives of the studies

described in this chapter are: (i) the *in situ* localization of the preprogalanin mRNA expression, and (ii) the quantification of the changes in preprogalanin mRNA expression in the telencephalon and hypothalamus of goldfish during feeding and starvation.

6.2. Materials and Methods

Animals

Male and female goldfish (*Carassius auratus*) of the common or comet varieties and approximately 40-50 g body weight were purchased from the Mount Parnell Fisheries (Mercersburg, PA, USA) and maintained under a simulated natural photoperiod of Edmonton, Alberta, Canada. Fish were fed once daily at a scheduled feeding time with pellet fish diet (Corey Aquafeeds, Fredericton, New Brunswick, Canada). Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, British Columbia, Canada) before decapitation and dissection of the brain. All protocols adhered to the policies of the Canadian Council of Animal Care and were approved by the Faculty of Science Animal Care Committee, University of Alberta.

Sampling plan for tissue collection

(a) Preprandial and postprandial experiment

Seven groups of fish (n = 5), all at a similar seasonal stage of sexual development were kept in 65 l glass aquaria under the same photoperiod and temperature conditions. Fish were fed daily at a scheduled time (1500 h) and allowed to acclimatize to the aquarium conditions and feeding time for a minimum of 8 weeks.

On the first sampling day, fish from one group each were sacrificed at 3 h (1200 h) and 1 h (1400 h) before the scheduled feeding time and at the scheduled feeding time (1500 h), and tissue samples (telencephalon and hypothalamus) collected. Two groups of fish remained unfed at the regular feeding time and were sampled at 1 h (1600 h) and 3 h (1800 h) after the scheduled feeding time and tissues were collected. On the second sampling day, two other groups of fish were fed at the scheduled feeding time (1500 h) and sampled at 1 h (1600 h) and 3 h (1800 h) after feeding. Tissues were stored at $^{-}80^{0}$ C until RNA extraction. In previous studies in our laboratory, preprandial and postprandial changes in NPY (Narnaware et al., 2000) and preproghrelin mRNA expression (Chapter 3) were detected in goldfish 3 h prior to and 3 h after the scheduled feeding time.

(b) Starvation experiment

Four groups of fish (n = 8) were acclimatized as described in the pervious section. One group was sampled (for collecting the telencephalon and hypothalamus) on day 1, after 24 h starvation (same as 0 h sample in the feeding experiment). The other three groups remained unfed and tissues were collected on days 3, 5 and 7 at 1700 h. Tissues were stored at $^{-}80^{0}$ C until RNA extraction.

Tissue preparation for in situ hybridization

In situ hybridization experiments were carried out as described previously (Cerda-Reverter et al., 2003). Fish were anesthetized in MS-222 and perfused transcardially with 60 ml of 0.65% physiological saline followed by a perfusion with the same volume of 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4).

Fish were decapitated and brains were removed and postfixed in 15 ml of the same fixative at 4 C. Next day, the tissues were dehydrated and embedded in paraffin (Paraplast, St. Louis, Missouri, USA). Transverse serial sections were cut at 10 μm using a rotary microtome and a set of four sections (40 μm) were mounted on 3-aminopropyltriethoxylane (TESPA)-treated slides. Every fifth section was eliminated and 12 slides covering the entire region of goldfish brain were considered to be a series. Slides were kept for a minimum of 24 h in a slide box with Drierite.

Prior to hybridization, the sections were deparaffinized, rehydrated and postfixed in 4% PAF for 20 min. Slides were then rinsed twice in PB for 5 min. After that the slides were treated with proteinase K solution (20 pg/ml in 50 mMTris –HCl, 5mM EDTA, pH 8) for 5 min at room temperature. Slides were then washed with PB and again fixed in PAF for 5 min, followed by washes in sterile water and triethanolamine (0.1 M, PH 8)/ acetic anhydride solution. Slides were then dehydrated and dried at room temperature.

Riboprobe synthesis and labeling

Full length preprogalanin 1B cDNA (GenBank Accession number AF416925; Chapter 6) was inserted into pGEM-T vector (Promega, Madison, Wisconsin, USA) and was used for making the riboprobes by *in vitro* transcription using an *in vitro* transcription system (Promega). The preprogalanin 1B cDNA encodes the galanin mature peptide that has an insertion into the mature peptide region (Chapter 6). This probe covers the nucleotide sequences of three alternative splice variants encoded by goldfish galanin gene I (Chapter 6). Therefore, this probe

detects the three transcripts present in the goldfish brain. Anti- sense and sense strands were labeled with 5 µl of ^{35}S-UTP (20 mCi/ml, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) using the riboprobe system kit (Promega) according to the manufacturer's protocol. The templates were then digested with RQ1-DNase (Promega) for 15 min at 37 C and then incubated at ¯20 C for 1-2 h with 10 mg/ml of yeast RNA type III in 8% formamide. The ^{35}S-UTP riboprobes were purified using G-50 Sephadex Quick Spin columns (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) and used immediately for hybridization.

Hybridization

Series of 12 slides each were used for hybridization with the sense (control) and antisense probes for preprogalanin 1B. Briefly, the ^{35}S-UTP riboprobes for sense and antisense strands were incubated at 80^0 C for 5 min and diluted in hybridization buffer (50% formamide, 300 mM NaCl, 20 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 10% dextran sulphate, 1 X Denhardt's solution and 0.5 µg/ml yeast RNA type III) to obtain a final concentration of $2x10^4$ cpm/µl. 60 µl of this hybridization buffer containing the sense or antisense probes was added to the pretreated slide, covered with a cover slip and incubated overnight in a humidified chamber set at 55 C. The next day, the slides were taken out and incubated in 5X standard saline citrate (SSC; 1X SSC contains 150 mM NaCl, 15 mM sodium citrate, pH 7) at 55 C for 30 min to remove the coverslips.

The slides were then washed once in 2X SSC, 50% formamide, 10 mM DTT at 65 C for 30 min and three times in NTE buffer (500 mM NaCl, 5 mM EDTA, 10 mM Tris

HCl, pH 7.5). Slides were then treated with RNase (20 µg/ml in NTE buffer) for 30 min at 37 C followed by three rinses with NTE buffer for 10 min at 37^0 C. The slides were then washed once with 2X SSC, 50 % formamide, 10 mM DTT for 30 min at 65 C, once in 2X SSC at RT for 15 min and twice in 0.1 X SSC at RT for 15 min. The slides were then dehydrated in ethanol solutions containing 0.3 M ammonium acetate and dried at room temperature.

Signal detection

The slides were coated with Hypercoat photographic emulsion (Amersham Pharmacia Biotech) and exposed at 4^0 C under dry conditions for 30 days, developed in Kodak D-19 and counterstained in toluidine blue. Anatomical localization and nomenclature were based on the goldfish brain atlas (Peter and Gill, 1975).

Image analysis

All images were photographed using a Nikon DXM 1200 digital camera (Nikon Corporation, Tokyo, Japan) fitted to a Leica DM IRBE microscope (Leica Microsystems, Wetzlar, Germany). The size, contrast and brightness of the digital images were formatted using Photoshop (Adobe System Inc., San Jose, California, USA).

Slot blot analysis

Total RNA extraction

Total RNA was extracted from the telencephalon and hypothalamus samples stored at ¯80 C using Trizol reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada). The integrity of the RNA was determined using a

spectrophotometer at 260/280 nm. The RNA samples were kept at -80 C until the slot blot analysis was carried out.

Slot blot analysis of the experimental samples

From our preliminary studies, we found that 5 μg of total RNA from the telencephalon and hypothalamus is an appropriate quantity for slot blot analysis of the preprogalanin mRNA expression in the goldfish brain. 5 μg total RNA from the experiment samples (telencephalon and hypothalamus) were blotted into a Hybond Nylon membrane (Amersham Pharmacia Biotech) using the Bio-Dot SF blotting apparatus (Bio-Rad Laboratories, Hercules, California, USA). Specific cDNA probe covering the full length of goldfish preprogalanin 1B (hereafter will be referred as preprogalanin) was amplified as described previously (Chapter 6). The probes were labeled with [α-^{32}P] dCTP using a Rediprime II – Quickprime kit (Qiagen Inc., Mississauga, Ontario, Canada) following the manufacturer's protocol. The labeled probe was then purified using a QIAquick nucleotide removal kit (Qiagen) following the manufacturer's instructions. 10^6 cpm/ml of probe were used for hybridization. Hybridization was performed using methods described by Unniappan et al. (2003). After hybridization overnight at 65 C, the membranes were washed twice with washing solution (0.04 M $NaHPO_4$, 1mM EDTA, and 1% SDS) at 65 C. The membranes were then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, California, USA) for 7 days and the screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). As an internal control, the

membranes were stripped and reprobed with a [α-^{32}P] dCTP labeled goldfish β-actin (GenBank accession number AF079831) probe. The control and experimental samples from each experiment were blotted on the same nylon membrane to avoid variability.

Data analysis

Data are represented as mean ± SEM. Statistical analyses were conducted using ANOVA followed by Tukey's test. The preprogalanin mRNA levels were expressed as a ratio of the preprogalanin mRNA to β actin mRNA and then normalized as a percentage of the expression levels at $^{-}$3 h (for the preprandial/postprandial experiment) or the expression levels in day 1 (for the starvation experiment). The postprandial ($^{+}$1 h and $^{+}$3 h) preprogalanin mRNA expression was compared with the values of the corresponding unfed group using Independent t-test. Significance was considered at $p < 0.05$.

6.3. Results

In situ localization of preprogalanin mRNA

Preprogalanin mRNA was found in the goldfish telencephalon, preoptic area and the hypothalamus. In the telencephalon, preprogalanin mRNA expression was localized in the ventral part of the ventral telencephalon (Vv) (Figure 6.1.A). In the preoptic area, the nucleus preopticus paraventricularis (NPP) has prominent preprogalanin mRNA expression (Figure 6.1.B). Within the hypothalamus, preprogalanin mRNA was found in the lateral part of the lateral tuberal nucleus (NLTl) (Figure 6.1.C) and medial aspect of the lateral recessus nucleus (NRL)

(Figure 6.1.D-E). No preprogalanin mRNA signals were detected in other regions of the brain. No signal was detected by hybridization with the sense probes (Figure 6.1.F).

Preprandial and postprandial changes in preprogalanin mRNA expression

No changes in preprandial preprogalanin mRNA expression were found in the telencephalon (Figure 6.2.A) and the postprandial expression levels (Figure 6.2.A) were similar to preprandial levels. However, in unfed fish the preprogalanin mRNA expression in the telencephalon (Figure 6.2.A) significantly increased at 1 h and 3 h after the regular feeding time. Preprogalanin mRNA expression levels in the telencephalon (1 h and 3 h; Figure 6.2.A) of fed fish were significantly lower than the expression levels in unfed fish at the same time points.

No changes in preprandial preprogalanin mRNA expression were found in the hypothalamus (Figure 6.2.B) and the postprandial preprogalanin mRNA expression levels were similar to preprandial levels. However, in unfed fish, the preprogalanin mRNA expression in the hypothalamus at 3 h after the regular feeding time was significantly elevated compared to -3 h preprandial levels (Figure 6.2.B). Preprogalanin mRNA expression levels in the hypothalamus (3 h; Figure 6.2.B) of fed fish were significantly lower than the expression levels in unfed fish at the same time point.

Starvation induced changes in preprogalanin mRNA expression

Preprogalanin mRNA expression levels in the telencephalon and hypothalamus were unchanged on days 3, 5 and 7 of starvation compared to expression levels on day 1 [24 h starved fish] (data not shown).

6.4. Discussion

Our *in situ* hybridization studies provide evidence for preprogalanin mRNA expression in the telencephalon, preoptic area and hypothalamus of goldfish. Preprogalanin mRNA is expressed in the V*v*, NPP, NLT*l* and NRL of the goldfish brain. A wider distribution of preprogalanin mRNA in the rat brain has been demonstrated using *in situ* hybridization (Ryan and Gundlach, 1996). In rats, high levels of preprogalanin mRNA were found in the anteroventral preoptic, supraoptic, paraventricular and dorsomedial nuclei of the hypothalamus, while, low levels of preprogalanin mRNA expression were found in the periventricular and arcuate nuclei of the hypothalamus, dorsal raphe and dorsal cochlear nuclei, piriform cortex, medial septum and the retrochiasmatic area (Ryan and Gundlach, 1996). The teleostean homologues of mammalian brain nuclei are not well characterized. V*v* is considered as the teleost homologue of the mammalian septal nucleus (Northcutt, 1995). Similarly, the NLT of goldfish is considered as the homologue of mammalian arcuate nuclei (Peng et al., 1994; Cerda-Reverter et al., 2003). Our results are in strong agreement with the immunohistochemical localization of galanin in the goldfish brain (Rao et al., 1996). The largest number of galanin-immunoreactive perikarya was found in the NPP of goldfish. A large aggregation of galanin-

immunoreactive neurons was also found in the NLT. Isolated perikarya were also found in the NRL (Rao et al., 1996). In the current study, we found preprogalanin mRNA expression in all these regions and the strongest expression was found in the NPP. NPP and NLT neurons innervate the goldfish pituitary, suggesting a role for these neurons in the regulation of pituitary hormone release (Anglade et al., 1993; Rao et al., 1996). Galanin stimulates both GH and gonadotropin (GtH-II) secretion from the goldfish pituitary (Rao et al., 1996). Galanin mRNA expression in the telencephalon and hypothalamus, the brain regions regulating food intake, suggests a possible role for galanin in the regulation of food intake in goldfish.

NPY-immunoreactive perikarya and fibers (Pickavance et al., 1992), and AgRP (Cerda-Reverter and Peter, 2003b) and MC-4R (Cerda-Reverter et al., 2003) mRNA are present in the NLT of the goldfish brain, where preprogalanin mRNA expression is also found. NPY mRNA is also expressed in the NPP of goldfish (Peng et al., 1994). Preprocholecystokinin (ppCCK) mRNA is expressed in the NRL of goldfish brain (Peyon et al., 1998), where we found the expression of preprogalanin mRNA. This potential colocalization suggests possible interactions of galanin with the NPY/AgRP system and ppCCK in regulating food intake in goldfish. Recently, Bergonzelli et al. (2001) demonstrated that galanin acts directly on the hypothalamic NPY neurons of rats to stimulate NPY release, suggesting that NPY is involved in the galanin signaling in the hypothalamus. Central administration of galanin stimulates food intake in goldfish (de Pedro et al., 1995; Volkoff and Peter, 2001). These results provide further evidence for the orexigenic

and hypophysiotropic actions of galanin in goldfish. Our results suggest that the distribution of galanin is evolutionarily conserved. Previously, we have detected preprogalanin mRNA expression in the telencephalon, hypothalamus, midbrain and the olfactory bulbs and tracts of goldfish, using Northern blot analysis on total RNA extracted from tissue pooled from several fish (Chapter 6). In the present study, using *in situ* hybridization preprogalanin mRNA expression was detected only in the telencephalon and hypothalamus of goldfish. The fact that we were using a pool of tissues in our previous study likely explains why signals were not detected in a wider range of brain areas in the present study.

To the best of our knowledge this is the first report on the changes in preprogalanin mRNA expression in the brain of any species during the scheduled feeding time. The postprandial preprogalanin mRNA expression in the telencephalon and hypothalamus of the fed group was significantly lower than the unfed group. In goldfish, a preprandial increase and a postprandial decrease in NPY mRNA expression in the brain has been demonstrated (Narnaware et al., 2000). Postprandial preproghrelin mRNA expression and serum ghrelin levels in fed goldfish were significantly lower than the unfed goldfish (Chapter 3). Although we did not find a preprandial increase in galanin mRNA expression in the goldfish brain, a postprandial decrease in galanin, suggests that galanin may take part in the regulation of food intake in goldfish. These results support the previous studies that found an increase in food intake in goldfish, immediately following the central administration of galanin (de Pedro et al., 1995; Volkoff and Peter, 2001).

Preprogalanin mRNA expression in the goldfish brain remains unchanged during 7 days of starvation. Our results agree with similar studies that did not find any change in preprogalanin mRNA expression in the rat brain after 48 h (Schwartz et al., 1993) or 72 h (Mizuno et al., 1998) starvation. Several studies reported the effects of prolonged starvation in teleost fish (Wootton, 1990; Rueda et al., 1998; Boujard et al., 2000; Gaylord and Gatlin III, 2000; Gaylord et al., 2001). Prolonged starvation changes several aspects of foraging behavior in teleost fish (Wootton, 1990). This includes an increase in feeding attempts and the attempt to eat inedible objects. After a four day starvation, the total amount of food eaten in the first hour of refeeding is significantly increased (Wootton, 1990). A hyperphagic phase following 10 weeks starvation was found in channel catfish, *Ictalurus punctatus* (Gaylord and Gatlin, 2000). No hyperphagia was found in channel catfish following 1 week starvation (Gaylord et al., 2001). Refeeding, following starvation results in hyperphagia in red porgies *[Pagrus pagrus]* (Rueda et al., 1998). All these evidences suggest that prolonged starvation could result in increased hunger and appetite in fish. Seven days of starvation increases NPY (Narnaware et al., 2000), AgRP (Cerda-Reverter and Peter, 2003b) and ghrelin mRNA expression (Chapter 3) in the goldfish brain. Serum ghrelin levels also increase on days 3 and 5 of starvation (Chapter 3). An increase in the mRNA expression and serum levels of orexigens may contribute to the hunger drive and appetite in fish. Based on our current results, it is possible to suggest that galanin may not be a key player in the regulation of food intake after prolonged starvation in goldfish.

In summary, this study found the expression of preprogalanin mRNA in the brain nuclei that are involved in the regulation of food intake and pituitary hormone secretion in goldfish. This result, together with the meal related changes in preprogalanin mRNA expression levels provide evidence for the orexigenic actions of galanin in goldfish. Absence of any change in preprogalanin mRNA expression in the goldfish brain during prolonged starvation suggests that galanin may not play a role in the regulation of hunger and appetite during starvation.

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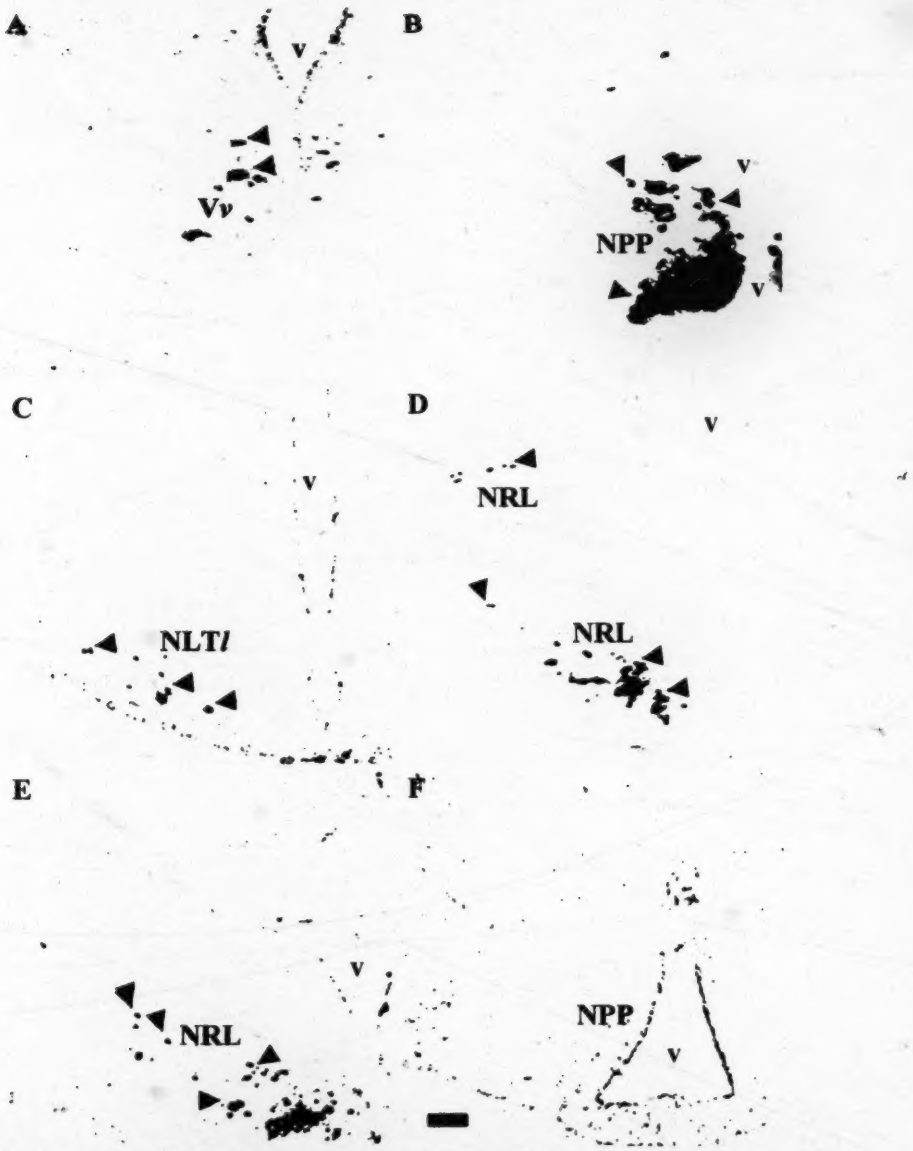

Figure 6.1. *In situ* hybridization of preprogalanin mRNA expression in the goldfish brain. Photomicrographs of transverse sections of goldfish brain at the levels of the telencephalon (Figure 6.1.A), preoptic area (Figure 6.1.B), and hypothalamus (Figures 6.1.C, D and E) showing expression of preprogalanin mRNA. Neurons expressing preprogalanin mRNA are indicated by arrows. Figure 6.1.F is a representative, control section (transverse section through the preoptic area) labeled with the sense probe. Vv = ventral telencephalon, NPP = nucleus preopticus paraventricularis, NLTl = lateral tuberal nucleus and NRL = lateral part of the nucleus recessus. Scale bar = 100 μm.

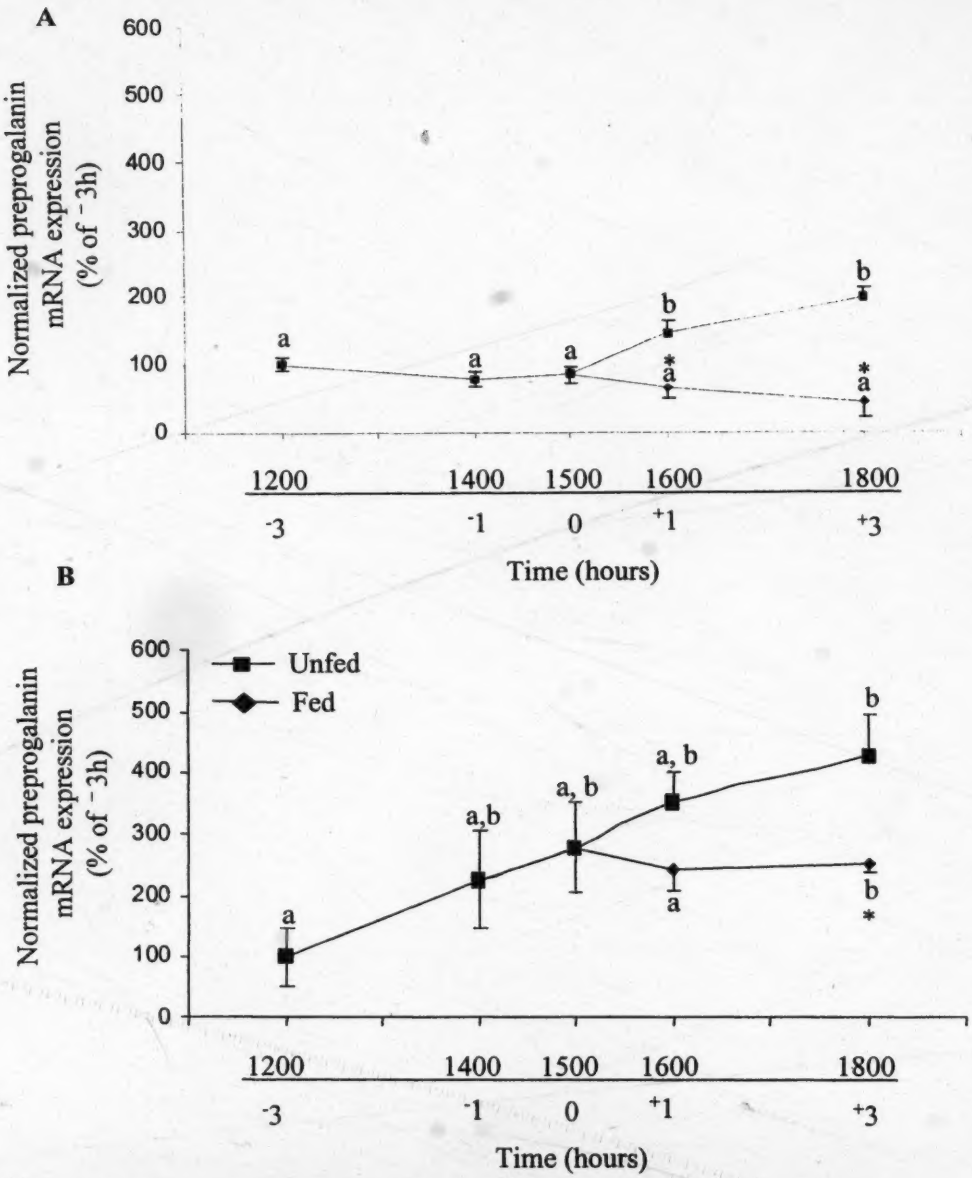

Figure 6.2. Preprandial and postprandial changes in the expression of preprogalanin mRNA in the telencephalon (Figure 6.2.A) and hypothalamus (Figure 6.2.B) of goldfish. Pre- and postprandial mRNA expression data represented as the normalized percentage of the preprogalanin mRNA expression in the -3 group (3 hours before the scheduled feeding time). 1500 h (0 h) is the scheduled feeding time. Data is presented as mean±SEM. n = 5 fish/group. Same letters denote values that are not statistically different. Asterisks represent significant differences between groups at the same time point.

CHAPTER 7

GENERAL DISCUSSION

Ghrelin and galanin are peptidyl endocrine factors that regulate many physiological functions in vertebrates. The main objectives of the research projects undertaken during my Ph.D. program were to study the structure, distribution and the physiological functions of ghrelin and galanin in goldfish. In this thesis, I have described my major findings of the studies conducted on ghrelin (Section A - Chapters 2 to 4) and galanin (Section B-Chapters 5 and 6) in goldfish. In this Chapter, a brief summary and discussion of the major findings of the projects on ghrelin and galanin are provided.

SECTION A – GHRELIN

Structure of ghrelin

Ghrelin is a recently discovered gut/brain hormone, first isolated from humans, rats and mice (Kojima et al., 1999). The peptide structure known so far indicates that the N-terminal region of ghrelin is highly conserved (GSSFLSP) (Kojima et al., 1999), except for the bullfrog ghrelin in which it is GLTFLS (Kaiya et al., 2002). Ghrelin has a unique, acyl modification in the third amino acid, serine, at the N-terminal region. Octanoyl group is the acyl group commonly found in ghrelin peptides (Kojima et al., 1999). However, other modifications such as decanoyl and decenoyl groups are also present in some species. The acyl modification of the third amino acid is critical for most physiological functions of ghrelin (Kojima et al., 1999; Thompson et al., 2003)

One of the major findings of the ghrelin project (Section A) is the identification of the structure of ghrelin cDNA and the organization of ghrelin gene in goldfish. Using 3'/5' RACE, RT-PCR and genomic DNA PCR, the cDNA structure of ghrelin in goldfish, and the gene organization of ghrelin from a non-mammalian vertebrate for the first time (Chapter 2) was successfully identified. The amino acid sequence obtained from the nucleotide sequence using bioinformatics tools indicates that the goldfish ghrelin has certain unique features. There are two putative cleavage sites and amidation signals (one after 12 amino acids and the second after 19 amino acids) in the mature peptide region of goldfish ghrelin, which suggests the presence of two amidated ghrelin (12 [$gGRL_{[1-12]}$]and 19 [$gGRL_{[1-19]}$] amino acids long) peptides in the goldfish. To the best of my knowledge, this was the first report of amidated forms of ghrelin in any species. In goldfish ghrelin, the second amino acid in the N-terminal region, serine [S] has been substituted with threonine [T]. Recently, the structure of ghrelin cDNA and peptide has been reported from a number of non-mammalian vertebrates including the chicken (Kaiya et al., 2002a), bullfrog (Kaiya et al., 2002b), frog (Galas et al., 2002), eel (Kaiya et al., 2003a), tilapia (Kaiya et al., 2003b, Parhar et al., 2003) and the rainbow trout (Kaiya et al., 2003c). Figure 7.1 shows the phylogenetic relationship of goldfish preproghrelin peptide with other known preproghrelin peptides (The phylogenetic tree was created using the Tree Graph/Phylogenetic tree options of the ClustalW program online at www.ebi.ac.uk/clustalw, using default parameters. The sequences of preproghrelin peptides from several species used for creating the tree were

obtained from the GenBank.). This figure shows the evolutionary relationship of goldfish ghrelin to other mammalian and non-mammalian ghrelin peptides. The grouping together of preproghrelin peptides from fish suggests the structural and evolutionary relationship of goldfish preproghrelin to other fish preproghrelins. The length of a line on the tree branches represents the amount of change in the preproghrelin peptide sequences in various organisms. The longer the line between two organisms, the greater the difference between their sequences. The length of the line in a tree is not an indication of time. It only represents the degree of change between the sequences.

Although the ghrelin peptides from goldfish were not purified, previous reports have shown that the prepropeptides having two putative cleavage sites and amidation signals result in the production of two peptides (eg: orexin A and orexin B, Sakurai et al., 1999). Another fact that is not clear from this study is the acyl modification present in goldfish ghrelin. In all species from which ghrelin has been identified, the octanoyl group is the major form of acyl group found in ghrelin. In some species, in addition to the octanoyl ghrelin, other forms of acylations including decanoylation and decenoylation are also present (Kaiya et al., 2002, Kaiya et al., 2003). It was assumed that octanoylation is the acyl modification present in the goldfish ghrelin peptides. Although the consensus is that the acyl modification is necessary for the biological actions of ghrelin, recent reports suggest that des-acyl ghrelin is active in stimulating adipogenesis in rats (Thompson et al., 2003). This finding challenges the notion that only acylated ghrelin is biologically active.

Synthetic octanoylated ghrelin peptides were used for the physiological studies presented in this thesis (Chapters 2-4). Based on the results of these studies, it is possible to state that the octanoylated forms of goldfish ghrelin peptides are biologically active in goldfish. Although these results do not indicate the type of acylation present in the goldfish, they do support our hypothesis that octanoylation may be the common acyl modification present in goldfish ghrelin peptides.

The human ghrelin gene organization was known (Wajnrajch et al., 2000) at the time of the identification of ghrelin gene organization in the goldfish. The human ghrelin gene is comprised of three introns and four exons. The intron:exon organization of goldfish ghrelin is identical to the human gene (Chapter 2). Notably, introns in the goldfish ghrelin gene are very short (Chapter 2). In rats and mice an additional 19 bp non-coding exon is present (Tanaka et al., 2001). However, this non-coding exon was not found in the goldfish ghrelin gene. Recently, ghrelin gene structure has been identified from the tilapia (Parhar et al., 2003) and rainbow trout (Kaiya et al., 2003c). The intron:exon organization of ghrelin gene in tilapia and trout is identical to the goldfish ghrelin gene. These results support the results of the studies described in Chapter 2. From these results, it is evident that the ghrelin gene organization is highly conserved in fish.

Distribution of ghrelin

Using RT-PCR analysis, ghrelin mRNA expression was detected in various tissues of goldfish (Chapter 2). The highest levels of ghrelin mRNA expression are in the gut, spleen and gills of goldfish. Weak expression of ghrelin

mRNA is also found in the brain. Northern blot analysis found ghrelin mRNA expression only in the gut of goldfish (Chapter 2). This result suggests that gut might be the major site of ghrelin production in goldfish. In mammals, the strongest expression of ghrelin mRNA is in the stomach (Kojima et al., 1999). Similar results were recently reported from the chicken (Kaiya et al., 2002a), bullfrog (Kaiya et al., 2002b), Japanese eel (Kaiya et al., 2003a), tilapia (Kaiya et al., 2003b), and rainbow trout (Kaiya et al., 2003c). Techniques, other than RT-PCR analysis did not detect ghrelin mRNA expression in the brain of any species studied so far. In a preliminary study, ghrelin mRNA expression in the telencephalon, hypothalamus and gut of goldfish (as shown in Appendix 1) was detected using *in situ* hybridization. Using slot blot analysis, ghrelin mRNA expression was successfully detected in the telencephalon and hypothalamus of goldfish (Chapter 3). These results indicate that slot blot analysis can be used as an effective tool to quantify the expression of ghrelin mRNA expression in the goldfish. Although, there are some limitations for this technique (Chapter 3), it is important to note that a quantifiable signal was found in slots loaded with total RNA from the telencephalon and hypothalamus. The expression of ghrelin mRNA in the telencephalon and hypothalamus, the brain regions that are known to be involved in the regulation of pituitary hormone secretion and food intake, indicate that ghrelin is involved in the regulation of these physiological functions in goldfish. To the best of my knowledge, there are no published reports on the immunohistochemical localization of ghrelin in any fish species.

Orexigenic actions of ghrelin in goldfish

Ghrelin is an orexigen in mammals (Wren et al., 2000, 2001., Tschop et al., 2000., Nakazato et al., 2001). In humans, there is a preprandial increase and a postprandial decrease in serum ghrelin levels, indicating that ghrelin acts as a meal initiator in humans (Cummings et al., 2002). In the studies that were conducted during my Ph.D. thesis project, it was demonstrated that the central and peripheral administration of human ghrelin and goldfish ghrelin peptides stimulate food intake in goldfish (Chapters 2 and 3). In addition to providing evidence for the orexigenic actions of ghrelin in the goldfish, these results indicate that both $gGRL_{[12]}$ and $gGRL_{[19]}$ are biologically active *in vivo* in goldfish. Using slot blot analysis, the meal-related changes in ghrelin mRNA expression were detected in the brain and gut of goldfish (Chapter 3). There is a postprandial decrease in ghrelin mRNA expression in the hypothalamus and gut of goldfish. Fasting increased ghrelin mRNA expression in the hypothalamus and gut of goldfish on day 7 of starvation (Chapter 3). These meal-related changes in ghrelin mRNA expression in the brain and gut, support the view that ghrelin is involved in the regulation of food intake in goldfish. In addition, a radioimmunoassay that reportedly measures only the acylated form of ghrelin was validated, for quantifying the serum ghrelin levels in goldfish (Chapter 3). A significant postprandial reduction and fasting-induced increase in serum ghrelin levels were found in the goldfish. These results further support the results of ghrelin mRNA expression studies in goldfish. This is the first study to report the orexigenic actions of ghrelin in a non-mammalian vertebrate. The

specific sites of action of ghrelin in regulating food intake in goldfish are currently not known.

In mammals, the orexigenic actions of ghrelin are mediated through other orexigens, mainly NPY and AGRP (Nakazato et al., 2001). In mammals, GHS-R are present in the NPY and AGRP neurons. NPY or AGRP immunoneutralization, or NPY or AGRP receptor blockade are known to abolish the effects of ghrelin on food intake in mammals (Nakazato et al., 2001). Orexin, another appetite stimulatory peptide is also involved in mediating the orexigenic effects of ghrelin in mammals (Toshinai et al., 2003). In goldfish, many orexigens and receptors including NPY (Narnaware et al., 2001), galanin (de Pedro et al., 1995, Volkoff et al., 2001), orexin (Volkoff and Peter, 2001), melanocortin-4 receptor (MC4-R) (Cerda-Reverter et al., 2003a), AGRP (Cerda-Reverter and Peter, 2003b) have been demonstrated. Anorexigens demonstrated are cholecystokinin (CCK) (Himick and Peter, 1994; Volkoff and Peter, 2003), bombesin (BBS) (Himick and Peter, 1994), corticotropin releasing factor (CRF) (Bernier and Peter., 2001), cocaine and amphetamine regulated transcript (CART) (Volkoff and Peter, 2002) and leptin (Volkoff et al., 2003). It is also known that certain orexigens and anorexigens interact (eg; NPY mediates the orexigenic actions of galanin, CCK mediating the anorectic effects of leptin) to regulate food intake in goldfish (Volkoff and Peter, 2001, Volkoff et al., 2003). The structure and functions of appetite regulatory peptides are highly conserved in goldfish (Lin et al., 2000). The interactions of ghrelin with the above mentioned appetite regulatory peptides and the involvement of these peptides in

mediating the orexigenic effects of ghrelin in goldfish are currently not known. However, it is possible to speculate that ghrelin interacts with other peptides in the brain and one or more of the appetite regulatory peptides are involved in mediating the effects of ghrelin on food intake in goldfish. Figure 7.2 is a model showing the peptides acting on goldfish brain to regulate food intake.

Actions of ghrelin on pituitary hormone release

A surprising finding of the studies described in Chapter 4 is the LH-releasing effect of ghrelin in goldfish. Ghrelin stimulates LH release *in vitro* and *in vivo* in goldfish. The results of the *in vitro* perifusion and static incubation studies indicate that both ghrelin peptides are biologically active *in vitro* and they can act directly on the gonadotropes of goldfish. This indicates that ghrelin receptor(s) is present in goldfish gonadotropes. GHS-R antagonist, D-Lys^3-GHRP-6 inhibited the effects of ghrelin in stimulating LH release, indicating that the GHS-R, present in the goldfish gonadotropes mediates the direct effects of ghrelin on LH release. In the static incubation studies described in Chapter 4, the stimulatory effects of ghrelin peptides on LH release were lower compared to the effects of the same concentration of ghrelin peptides in stimulating LH release from perifused pituitary cells. This reduction in the effect of ghrelin on LH release from static culture might have happened due to the desensitization of receptors during the prolonged (2 h) incubation. Preliminary results obtained from perifusion studies indicate that goldfish ghrelin desensitizes its receptors in the goldfish gonadotropes and more than three hours are necessary for the receptors to return to control levels (Appendix II).

A recent report indicates that ghrelin rapidly desensitizes the GHS-R1a expressed in mammalian cell lines and receptors return to control level only after 6 hours (Camina et al., 2003). Both ICV and IP injections of ghrelin stimulate serum LH levels in goldfish (Chapter 4). It is assumed that IP administered ghrelin stimulates LH release through its actions in the pituitary. ICV administered ghrelin may also reach the pituitary or act indirectly through possible stimulation of gonadotropin releasing hormone (GnRH) release. Ghrelin stimulates LHβ mRNA expression in goldfish gonadotropes *in vitro* (Chapter 4). This result indicates that, in addition to the stimulation of LH release, ghrelin also stimulates LH synthesis in goldfish. In mammals, ghrelin has no effect on LH release *in vitro* or *in vivo* (Kojima et al., 1999, Tolle et al., 2001).

Previous studies have shown that many factors including the GnRH (Marchant et al., 1983), NPY (Peng et al., 1993), taurine (Trudeau et al., 1993), inhibin (Ge and Peter, 1992), activin (Ge and Peter, 1992), gamma amino butyric acid (GABA) (Trudeau et al., 1993a), serotonin (Somoza and Peter, 1991), sulfonylated cholecystokinin (CCK-8s, Himick and Peter, 1995) and dopamine (Chang and Peter, 1983) are involved in the regulation of LH secretion in goldfish. To the best of my knowledge, ghrelin is the first known gut/brain hormone that regulates LH secretion in goldfish. Ghrelin is a novel addition to the array of peptides that regulates LH release from goldfish gonadotropes. Figure 7.3 is a model showing the factors that regulate LH secretion from goldfish gonadotropes.

Ghrelin stimulates GH release *in vitro* and *in vivo* in goldfish (Chapter 4). The results of my *in vitro* studies indicate that ghrelin acts directly on the somatotropes in goldfish. Ghrelin receptor(s) might be present in the goldfish somatotropes. GHS-R antagonist, D-Lys^3-GHRP-6 did not inhibit the stimulatory effects of ghrelin on GH release, indicating that receptor(s) other than the GHS-R present in the goldfish gonadotropes mediate the effects of ghrelin on GH release. In the static incubation studies described in Chapter 4, the stimulatory effects of ghrelin peptides on GH release were lower compared to the effects of the same concentration of ghrelin peptides in perifused pituitary cells. This reduction in the effect of ghrelin on GH release from static culture might have happened due to the desensitization of receptors during prolonged (2 h) incubation. Preliminary results obtained from perifusion studies suggest that goldfish ghrelin desensitizes the receptors present in goldfish somatotropes and more than three hours are necessary to return to control levels (Appendix II). A recent report indicates that ghrelin rapidly desensitizes the GHS-R in mammalian cell lines and receptor function returns to normal levels only after 6 hours (Camina et al., 2003). Somatostatin-14 (SS) inhibits the effects of ghrelin on GH release *in vitro* from dispersed pituitary cells in static culture and perifusion (Chapter 4). These findings agree with the studies that show SS inhibition of the stimulatory effects of ghrelin on GH release in rats (Tannenbaum et al., 2003), pigs (Hashizume et al., 2003) and humans (Broglio et al., 2002). Both ICV and IP injections of ghrelin stimulates GH release in goldfish. It is assumed that IP administered ghrelin stimulates GH release through its actions in the pituitary. ICV

administered ghrelin may also reach the pituitary or act indirectly through the stimulation of growth hormone releasing hormone (GHRH) release or inhibition of somatostatin release. The results of the present study agree with the previous findings from mammals (Kojima et al., 1999, Wren et al., 2000) and non-mammalian vertebrates such as tilapia (Riley et al., 2002, Kaiya et al., 2003), eel (Kaiya et al., 2003), rainbow trout (Kaiya et al., 2003), bullfrog (Kaiya et al., 2002) and chicken (Kaiya et al., 2002) that indicate that ghrelin is a GH-releasing peptide. The studies described in Chapter 4 are the first to: (1) demonstrate the central effects of ghrelin on GH release in a non-mammalian vertebrate, (2) *in vitro* effects of ghrelin on GH mRNA expression in any species studied so far, (3) first study in a non-mammalian vertebrate that shows the inhibitory effects of SS on the stimulatory role of ghrelin on GH release, and (4) the first study that used an antagonist for the GHS-R to study the possible role of a GHS-R like receptor in mediating the effects of ghrelin on pituitary hormone secretion.

In goldfish, several factors are involved in the regulation of GH secretion. These include the stimulatory factors such as GnRH (Marchant et al., 1989), NPY (Peng et al., 1993a), growth hormone-releasing factor (GRF) (Vaughan et al., 1992), thyrotropin-releasing hormone (TRH) (Trudeau et al., 1992), dopamine (Wong et al., 1992) activin (Ge et al., 1992), inhibin (Ge et al., 1992), bombesin (BBS) (Himick and Peter, 1994), CCK-8s (Himick and Peter, 1995), PACAP (Wong et al., 1998), and nitric oxide (NO) (Uretsky et al., 2001) and inhibitory factors including somatostatin (Yunker et al., 2003), serotonin (Somoza and Peter, 1991), glutamate

(Trudeau et al., 1996) and norepinephrine (Yunker et al., 2000), The results described in Chapter 4 clearly indicate that ghrelin is a GH-releasing peptide in goldfish. Ghrelin produced from both the gut and brain is presumably involved in the regulation of GH release in goldfish. Figure 7.4 is a model showing the factors that regulate GH secretion from the goldfish somatotrope.

Ghrelin - A hypophysiotropic orexigen?

The physiology of food intake, growth and reproduction are interconnected. Food intake is essential for acquiring the essential nutrients for growth and providing the energy for reproduction. GH regulates the process of growth by its direct action or by acting through the insulin-like growth factor. LH regulates several important processes in the physiology of reproduction. The physiological studies discussed in the Section A of this thesis indicate that ghrelin is involved in the regulation of food intake (Chapters 2 and 3) and GH and LH secretion (Chapter 4) in goldfish. Ghrelin stimulates food intake in goldfish, thereby providing the essential nutrients for growth, and energy for reproduction. In addition, ghrelin acts directly on the pituitary to stimulate GH and LH synthesis and release in goldfish. The regular release of GH (eg: diurnal pattern of GH release) and LH (eg: pre-ovulatory LH surge) facilitates the proper functioning of the physiological machinery regulating growth and reproduction in goldfish. Based on the results described in this thesis, it is possible to suggest that ghrelin acts in a bimodal fashion, directly by regulating the GH and LH release and indirectly by providing essential nutrients and energy to facilitate growth and reproduction. I have

coined a new term, hypophysiotropic orexigen, to represent the actions of ghrelin in goldfish. Figure 7.5 summarizes the roles of ghrelin in the regulation of food intake, growth and reproduction.

The results of the studies on ghrelin (Chapter 2-4) support my hypotheses (Chapter 1) about the presence, distribution and role of ghrelin in the regulation of food intake and pituitary hormone secretion in goldfish.

Future research

In this project, using molecular and physiological techniques and *in silico, in situ, in vitro* and *in vivo* approaches, the structure, distribution and some physiological functions of ghrelin in goldfish were discovered. The results described in section A of this thesis contribute to better our understanding about the physiology and endocrinology of goldfish. The results of my studies on ghrelin lay the foundation for future studies on ghrelin in goldfish.

Some of the areas/topics of future studies on ghrelin in goldfish include the following: (1) **structure and distribution of the GHS-R and other receptors that mediate the functions of ghrelin in goldfish:** using molecular biology tools, including 3'/5' RACE, screening of goldfish cDNA library to obtain the structure of ghrelin receptors, and expression of the receptor in cell lines to study the signaling of the receptors would be of interest; (2) **routes of action of ICV ghrelin on GH and LH in release in goldfish:** currently, the mode of action of ghrelin in releasing GH and LH release, when injected into the brain is not clear; more detailed *in vivo* studies and mRNA expression studies are necessary to elucidate the sites of actions

of ghrelin; (3) **types of acyl modifications present in ghrelin peptides in goldfish:** purification of the ghrelin peptides using HPLC and identification of the acyl modifications present in goldfish would be a useful line of research; (4) **mechanisms and sites of acylation of ghrelin in goldfish:** currently the sites of acylation and mechanisms of acylation are not known; (5) **interactions of ghrelin with other hormones in regulating food intake and pituitary hormone secretion:** interactions of ghrelin with other peptides in regulating the GH and LH release and food intake in goldfish are not known, and accordingly it would be of interest to study the role of peptides, especially GHRH, GnRH and SS in mediating the effects of ghrelin on GH and LH release and NPY and AGRP in mediating the effects of ghrelin on food intake in goldfish; (6) **second messenger systems involved in the actions of ghrelin on GH and LH release:** as nothing is known about the signal transduction mechanisms that mediate the effects of ghrelin on GH and LH release in goldfish, *in vitro* pharmacological methods and calcium imaging could be used to study the second messenger system(s) involved in the actions of ghrelin on GH and LH release; (7) **role of ghrelin in the secretion of pituitary hormones other than GH and LH:** as the role of ghrelin on the regulation of the release of hormones other than GH and LH in goldfish is currently not known, it would be of interest to study the role of ghrelin on the release of other hormones from the pituitary; (8) **seasonal and diurnal changes in the expression of ghrelin mRNA and circulating levels of ghrelin in goldfish:** studying the role of steroid hormones in the regulation of ghrelin mRNA expression and the seasonal variations, if any, in ghrelin mRNA

expression and peptide production, would be of interest to help understand the role of ghrelin in the regulation of physiological functions in goldfish during various seasons; and (9) **the effects of macronutrients on ghrelin mRNA expression in the brain and gut, and serum ghrelin levels in goldfish:** the role of varying concentrations of nutrients, especially carbohydrate, protein and fat in the regulation of ghrelin synthesis and secretion is another area of interest for future research.

SECTION B – GALANIN

Multiple forms of galanin in goldfish

The most important finding of the studies described in Section B of this thesis is the identification of multiple forms mRNAs encoding putative galanin peptides in the goldfish (Chapter 5). Based on my RACE and genomic DNA PCR results, it is evident that two genes encoding galanin are present in the goldfish. There are three alternative splice variant mRNAs that form from galanin gene I, and two alternative splice variants that form from gene II. This is the first study (Chapter 5) to report the structure of galanin mRNAs from a fish and the first to report the gene organization of galanin in a non-mammalian vertebrate. Figure 7.6 is a scheme showing the formation of five forms of galanin in goldfish. Goldfish is tetraploid and the presence of two genes is not surprising. In goldfish, many other neuropeptides and hormones including GH (Chan et al., 1996), salmon and chicken GnRH (Lin and Peter, 1997), prolactin (Law et al., 1996) and CART (Volkoff and Peter, 2001) are encoded in duplicate genes.

In goldfish, the preprogalanin mRNAs encode five putative galanin peptides that are 29 (two peptides from preprogalanin 1A and 1C), 31, 53 or 55 amino acids long. In all five alternative transcripts of galanin in goldfish, the 1-13 amino acids are conserved. The N-terminal conserved region is known to be the receptor binding sequence in mammals (Iismaa and Shine, 1999). Hence, it is possible to speculate that all five transcripts in goldfish are biologically active. However, the structure of galanin receptors, affinity of the various ligands identified in this project and the

functional significance of the multiple ligands are currently not known. The findings described in Chapter 5 are supported by a recent study that reported alternative splice variants of galanin in Japanese quail (Kohchi and Tsutsui, 2000). In Japanese quail, two forms of galanin are present and the alternate transcript (long form) of galanin has a 69-nucelotide insertion in the region encoding the mature peptide galanin. This transcript has the insertion immediately after the conserved 1-13 amino acids in the N-terminal region.

Figure 7.7 is a phylogenetic tree showing the relationship of goldfish preprogalanin peptides with the preprogalanin peptides of mammals and quail (The phylogenetic tree was created using the Tree Graph/Phylogenetic tree options of the ClustalW program online at www.ebi.ac.uk/clustalw, using default parameters. The sequences of preprogalanin peptides from several species used for creating the tree were obtained from the GenBank.). This figure shows the evolutionary relationship of goldfish galanin peptide to other mammalian and non-mammalian peptides. The grouping of preprogalanin peptides from goldfish with the preprogalanin of Japanese quail suggests the structural and evolutionary relationship of goldfish preprogalanin with quail preprogalanin.

Galaninergic neurons in the goldfish brain – Physiological implications

This study found a tissue-specific expression of alternative transcripts in goldfish (Chapter 5), suggesting that galanin might have tissue specific roles in goldfish. In this study (Chapter 2), using *in situ* hybridization, galanin mRNA expression was detected in the ventral telencephalon (Vv), the nucleus preopticus

paraventricularis (NPP), the lateral tuberal nucleus (NLTl), and medial aspect of the lateral recessus nucleus (NRL). A previous study conducted in our laboratory had identified a wide distribution of galanin immunoreactivity in the goldfish brain and pituitary (Rao et al., 1996). NPP and NLT neurons innervate the goldfish pituitary, suggesting a role for these neurons in the regulation of pituitary hormone release (Anglade et al., 1993; Rao et al., 1996). Results of preliminary studies conducted in our laboratory indicate that galanin stimulates both GH and LH secretion from the goldfish pituitary (Peng C, 1993; Rao et al., 1996). Several studies have shown that the central administration of galanin stimulates food intake in goldfish (de Pedro et al., 1995; Volkoff and Peter, 2001). In the present study, galanin mRNA expression was found in brain nuclei that are involved in the regulation of food intake in goldfish (Chapter 6).

NPY immunoreactive perikarya and fibers (Pickavance et al., 1992), AGRP (Cerda-Reverter and Peter, 2003b) and MC4-R (Cerda-Reverter et al., 2003a) mRNAs are present in the NLT, NPY mRNA is present in the NPP (Peng et al., 1994) and preprocholecystokinin (ppCCK) mRNA is expressed in the NRL of goldfish brain (Peyon et al., 1998). In the present study (Chapter 6), expression of preprogalanin mRNA was detected in these regions of brain where other appetite regulatory peptides or mRNAs encoding these peptides or their receptors are present. The colocalization of galanin with NPY, AGRP, MC4-R and CCK suggests the possible interaction of galanin with these peptides or receptors in regulating food intake in goldfish. Volkoff and Peter (2001) found that galanin mediates the

orexigenic effects of NPY and vice versa. The results described in Chapter 5 provide neuroanatomical evidence for the results described by Volkoff and Peter (2001) and other similar studies on the hypophysiotropic and appetite regulatory functions of galanin in goldfish. A postprandial decrease in galanin mRNA expression in the telencephalon and hypothalamus (Chapter 6) provides further evidence for the orexigenic actions of galanin in goldfish.

The results of the studies described in section B of this thesis support my hypotheses (Chapter 1) that suggested the presence and wide distribution of galanin in goldfish, and meal related changes in galanin mRNA expression in the brain.

Future research

The results of the studies described in Chapters 5 and 6 of this thesis, together with the previous studies on the role of galanin in food intake and pituitary hormone secretion indicate that galanin is a multifunctional neuropeptide in goldfish. The discovery of multiple forms of galanin cDNAs and the brain mapping of galanin in goldfish described in Section B of this thesis provide more useful tools for further studies of galanin in goldfish. Some of the themes for future research on galanin include the following: (1) **functional significance of multiple galanin transcripts in goldfish:** currently the physiological significance of multiple forms of galanin are not known; detailed studies on the physiological functions of the native galanin peptides on food intake, pituitary hormone secretion and other functions in fish will give evidence for the relevance of the presence of multiple forms of galanin in goldfish; (2) **seasonal variations in the tissue specific expression of the multiple**

galanin mRNAs in goldfish: seasonal and tissue specific expression of galanin mRNAs using RNase protection assay and quantitative PCR would be another useful area of study; (3) **mechanisms of the *in vitro* and *in vivo* actions of galanin on pituitary hormone secretion**: the role of other peptides in mediating the actions of galanin on GH and LH release *in vivo* and the signal transduction mechanisms mediating the effects of galanin on pituitary hormone secretion would be a useful line of research; and (4) **the identification of galanin receptors**: using a combination of *in vitro* and *in vivo* approaches it would be possible to identify the structure and distribution of galanin receptors in goldfish and the affinity of the multiple ligands to these receptors.

Ghrelin and Galanin: Two peptides with some common physiological functions in goldfish?

The studies presented in this thesis have shown that ghrelin is a novel peptide involved in the regulation of food intake (Chapters 2-3) and GH and LH release (Chapter 4) in goldfish. Galanin has been shown to stimulate food intake (Volkoff and Peter, 2001) and GH and LH secretion in goldfish (Peng, C, 1993b). Thus, ghrelin and galanin are two peptides that have a common role in the regulation of pituitary hormone release and food intake in goldfish. There is a postprandial decrease in preproghrelin mRNA expression in the hypothalamus and gut of goldfish (Chapter 3). A similar, postprandial decrease in preprogalanin mRNA expression has been found in the telencephalon and hypothalamus of goldfish (Chapter 6). In goldfish, the telencephalon and hypothalamus are two brain regions that are

primarily involved in the regulation of food intake (Chapter 6). The presence of both ghrelin (Chapters 2-3 and Appendix 1) and galanin mRNAs (Chapters 5-6) in the telencephalon and hypothalamus of goldfish and a similar change in their mRNA expression pattern following food intake (Chapters 3 and 6) suggests the interaction of ghrelin and galanin in the regulation of food intake in goldfish. However, the results available at present do not provide enough evidence for the possible interactions of ghrelin and galanin in the regulation of any physiological functions in goldfish. Future studies must give attention to the possible interactions of galanin and ghrelin in the regulation of food intake and pituitary hormone secretion *in vivo* in goldfish.

7.3. REFERENCES

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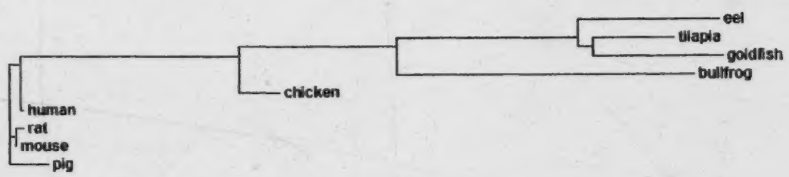


Figure 7.1. Phylogenetic tree showing the relationship of goldfish ghrelin peptides to the other known peptides.

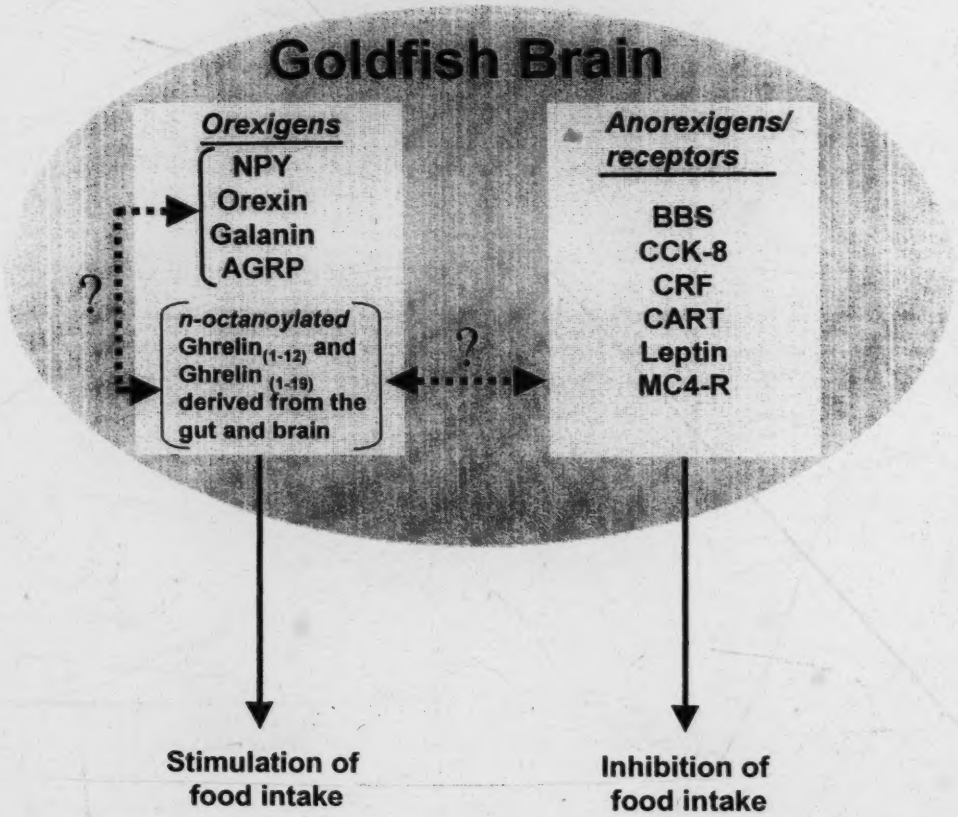


Figure 7.2. Model shows the peptides that act act on the goldfish brain to regulate food intake in goldfish. Broken lines with arrow heads show possible interactions of ghrelin with other appetite regulatory peptides.

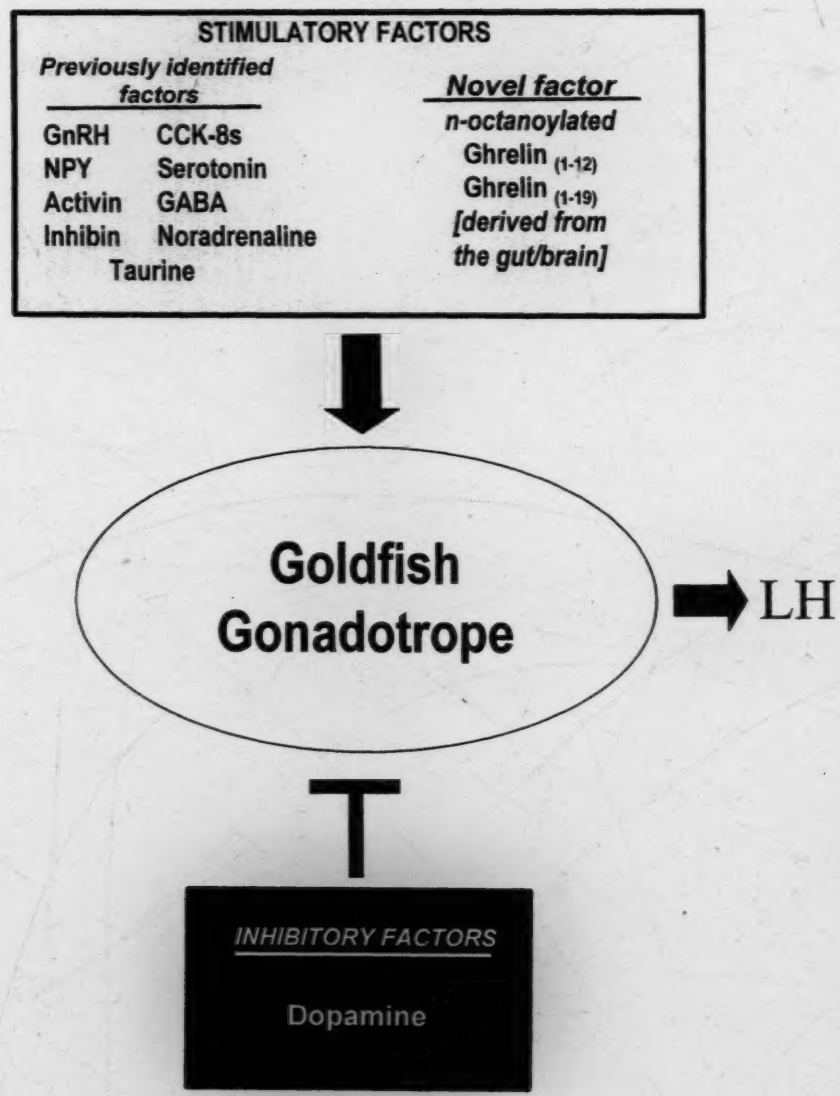


Figure 7.3. Model showing the factors that regulate LH release from the goldfish gonadotrope. Arrow heads indicate stimulation and blunt heads indicate inhibition.

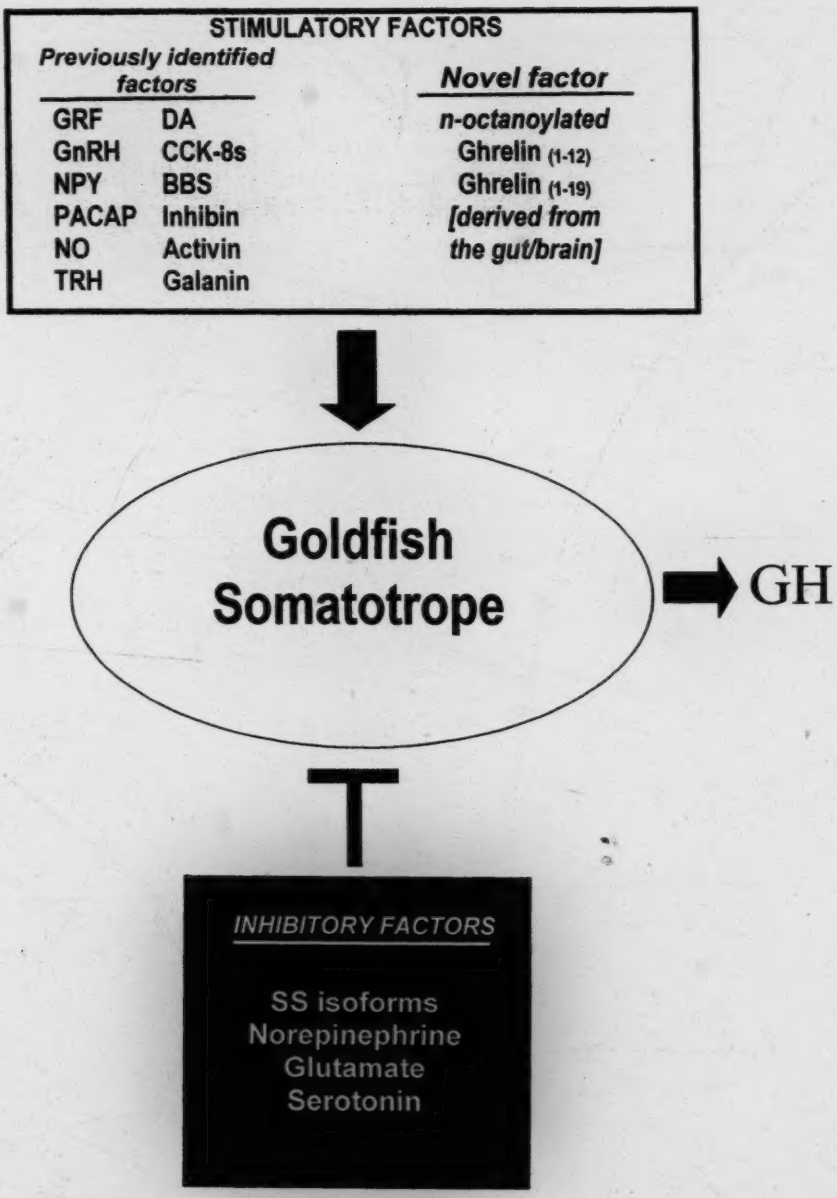


Figure 7.4. Model shows the factors that regulate GH release from the goldfish somatotrope. Arrow heads indicate stimulation and blunt heads indicate inhibition.

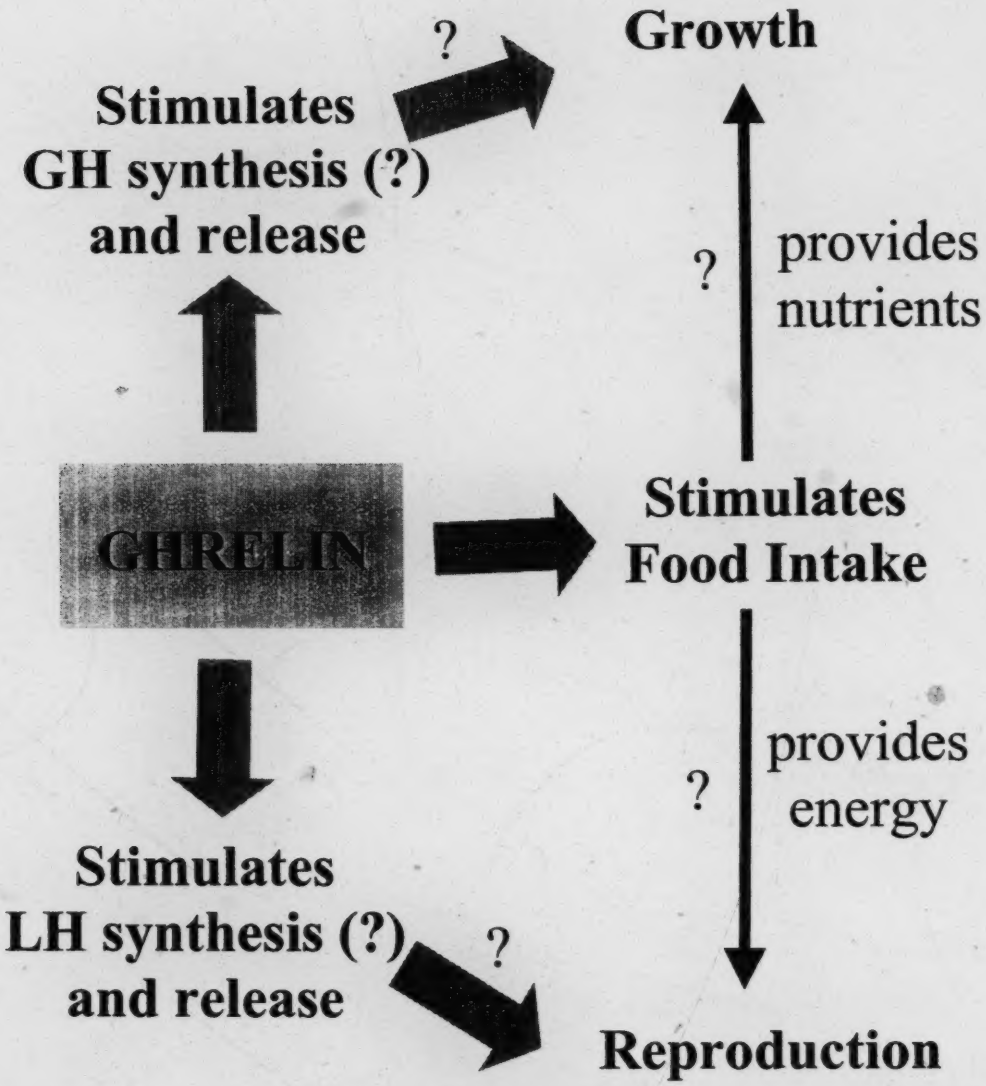


Figure 7.5. Possible roles of ghrelin in the regulation of food intake, growth and reproduction. The question marks indicate possible actions (that needs more experimental support) of ghrelin. Arrowheads indicate stimulation or positive influence.

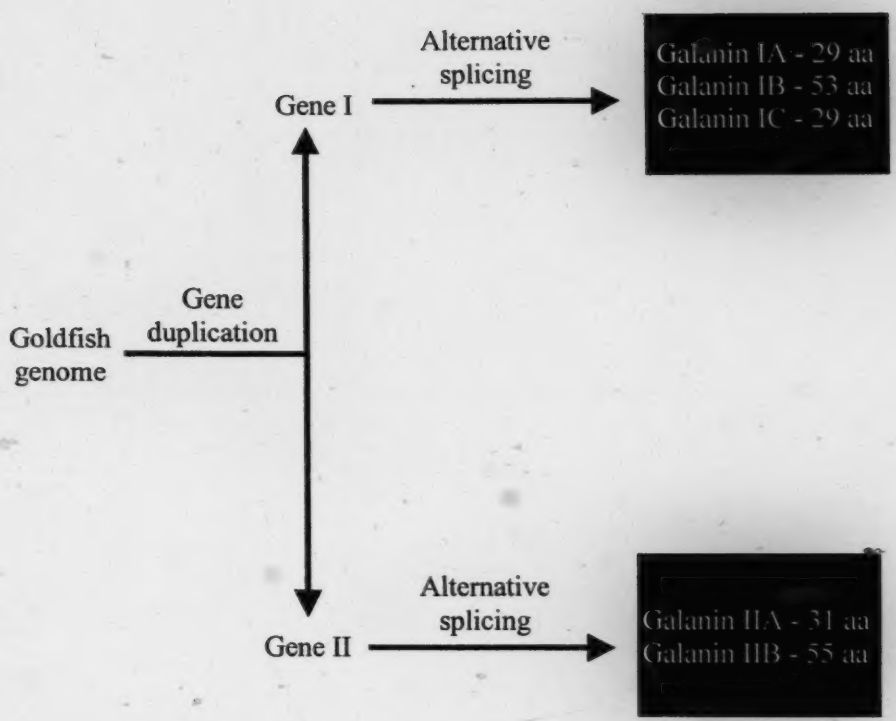


Figure 7.6. Scheme of formation of multiple forms of galanin peptide in goldfish.

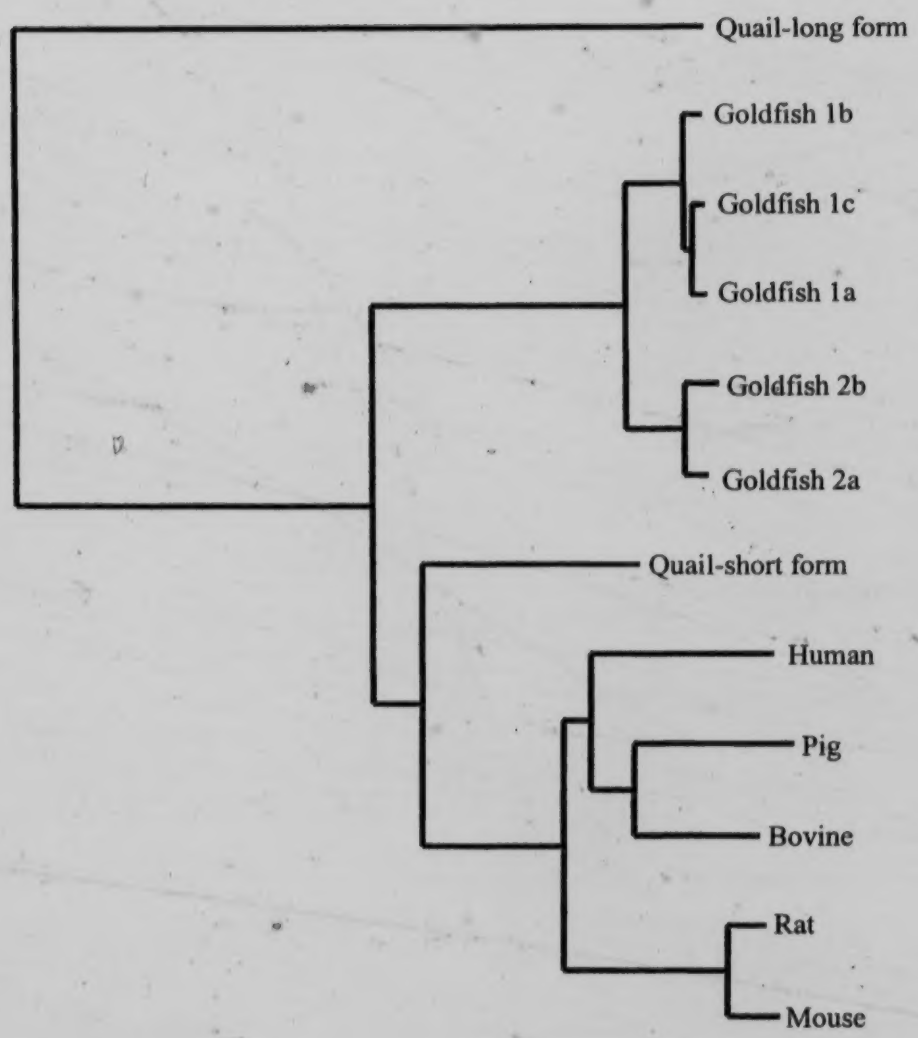


Figure 7.7. Phylogenetic tree showing the relationship of goldfish preprogalanin peptides to the some other known preprogalanin peptides.

APPENDIX I

IN SITU LOCALIZATION OF PREPROGHRELIN mRNA EXPRESSION IN THE BRAIN AND GUT OF GOLDFISH

Introduction

Ghrelin mRNA expression has been identified in the gut of mammals and non-mammalian vertebrates using RT-PCR analysis, Northern blot and *in situ* hybridization. However, there are no reports available on the identification of preproghrelin mRNA expression in the brain using Northern blot analysis or *in situ* hybridization.

In this study, *in situ* hybridization was used to detect the preproghrelin mRNA expression in the brain and gut of goldfish.

Methods

In situ hybridization was conducted as described in Chapter 6, except for the following changes. Goldfish gut sections of 10 micron thickness were taken from the 'J' region of the gut (described in Chapter 3). Goldfish preproghrelin (Chapter 2) riboprobe was synthesized as described in Chapter 6. The duration of exposure of the slides was 40 days.

Results and Discussion

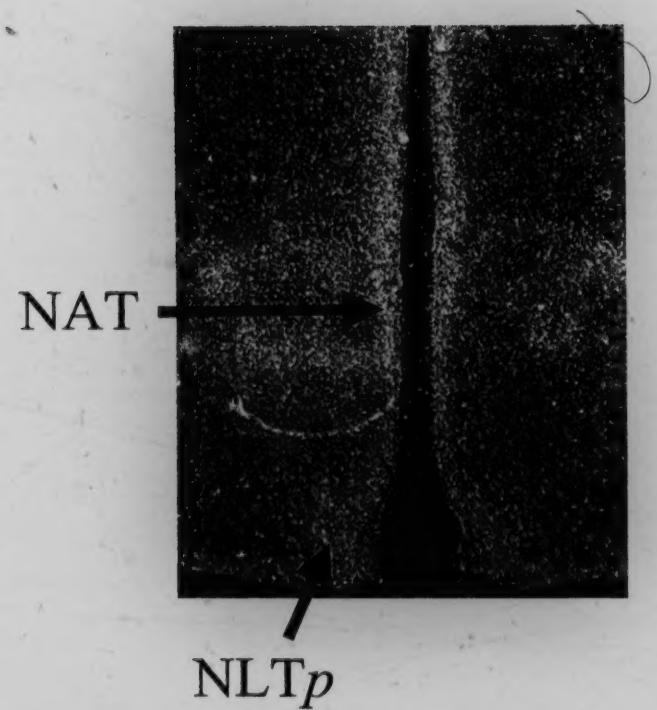
Ghrelin mRNA expression was detected in the area ventralis telencephali pars lateralis (V*l*) of the goldfish telencephalon (Figure A1.1). Weak expression of ghrelin mRNA was also detected in the nucleus anterior tuberis (NAT) and the

nucleus lateral tuberis pars inferioris (NLTp) of the goldfish hypothalamus (Figure A1.2). No signals were found in other regions of the brain. Strong signals of ghrelin mRNA expression were found in the gut of goldfish (Figure A1.3).

These results provide further support for the RT-PCR and slot blot analyses that found ghrelin mRNA expression in the brain and gut of goldfish (Chapters 2 and 3). The presence of ghrelin in the brain supports the central actions of ghrelin in the regulation of food intake and pituitary hormone secretion described in chapters 2, 3 and 4. Although it was possible to repeatedly localize ghrelin in the gut, the results from the *in situ* localization of ghrelin in the goldfish brain were not repeatable.

Vl = Area ventralis telencephali pars lateralis

Figure A1.1. Photomicrograph showing the expression of preproghrelin mRNA in the telencephalon of goldfish. Arrows indicate the preproghrelin mRNA signal.



NAT = nucleus anterior tuberis
NLTp = nucleus lateral tuberis pars inferioris

Figure A1.2. Photomicrograph (darkfield) showing the expression of preproghrelin mRNA in the hypothalamus of goldfish. Arrows indicate the preproghrelin mRNA signal.

Figure A1.3. Photomicrograph (darkfield) showing the darkfield images of preproghrelin mRNA expression in the gut of goldfish. Arrows indicate the preproghrelin mRNA signal.

APPENDIX II

DOES GHRELIN DESENSITIZE ITS RECEPTOR(S) IN GOLDFISH SOMATOTROPES AND GONADOTROPES?

Introduction

Ghrelin stimulates GH release *in vitro* and *in vivo* in mammals and non-mammalian vertebrates. In section A, chapter 4, I have shown that ghrelin stimulates GH and LH release *in vitro* and *in vivo* goldfish (Chapter 4). GHS (Orkin et al., 2003) and ghrelin (Camina et al., 2003) has been found to desensitize the GHS-R in mammals. Preliminary evidence for the desensitization of receptors of ghrelin in goldfish pituitary cells by human ghrelin and goldfish ghrelin peptides was obtained from the following experiments.

Methods

Dispersion of pituitary cells and perifusion were conducted as described in Chapter 5.

Experiment I

Following the four hour perifusion with plain testing media to stabilize the basal levels of GH and LH, the first 5-minute pulse of test solution containing hGRL (1000 nM) was administered. Subsequently, a 5-minute pulse of the testing medium containing the specified concentration (100, 10, 1, or 0.1 nM) of hGRL was administered at 60 minute intervals. 60 minute after the last pulse of hGRL (0.1 nM), a 5-minute pulse of 100 nM sGnRH, a potent stimulator of GH secretion in

goldfish was administered as the positive control. Perifusate fractions were collected and stored at –20 C until GH RIA.

Experiment II

Following the four hour perifusion with plain testing media to stabilize the basal levels of GH and LH, a 5-minute pulse of $gGRL_{19}$ (0.01, 0.1 or 1 nM) was administered into one column. The columns were then perifused for 3 hours with plain testing media, at which time, a single pulse of the same concentration (0.01, 0.1 or 1 nM) of $gGRL_{19}$ was administered. Perifusate was collected and stored at –20 C until GH and LH RIA.

Experiment III

Following the four hour perifusion with plain testing media to stabilize the basal levels of GH and LH, a 5-minute pulse of $gGRL_{12}$ (1000 nM) or $gGRL_{19}$ (1000 nM) was administered to each perifusion column. The columns were then perifused for 90 minutes with plain testing media, at which time a single pulse of the 100 nM sGnRH was administered into each column. Perifusate was collected and stored at –20 C until GH and LH RIA.

Results

Experiment I

Figure A2.1 shows the profile of GH release from goldfish pituitary cells treated with descending concentrations of hGRL. In columns perifused with descending concentrations, the first 5-minute pulse of 1000 nM hGRL significantly stimulated GH secretion from pituitary cells (Figure A2.1.A and B). No significant

increase in GH levels was found after the administration of lower concentrations of hGRL and 100 nM sGnRH (Figure A2.1.A and B).

Experiment II

The first 5-minute pulse of all concentrations (0.1, 1 and 10 nM) of $gGRL_{19}$ stimulated GH (Figure A2.2. left panel) and LH (Figure A2.3. left panel) release from the dispersed pituitary cells of goldfish. However, the second 5-minute pulse of the same concentrations of $gGRL_{12}$ and $gGRL_{19}$ did not cause any effect on GH (Figure A2.2, right panel) or LH (Figure A2.3, right panel) release.

Experiment III

No effects were found for 1000 nM $gGRL_{12}$ and $gGRL_{19}$ on GH (Figure A2.4. A, B) and LH (Figure A2.5. A, B) release from goldfish pituitary cells. 100nM sGnRH administered 90 minutes after the first pulse stimulated GH (Figure A2.5. A, B) and LH (Figure A2.5. A, B) release from the same columns treated with goldfish ghrelin peptides.

Discussion

Experiment I

The most important finding of this experiment is the effect of a single treatment of a high dose of hGRL in inhibiting the response of the somatotropes to other following doses of hGRL administered at 60 minute intervals. A single administration of 1000 nM stimulates GH secretion and inhibits the effect of next four doses of hGRL applied at 60 minute intervals. In these columns where 1000 nM was administered first, sGnRH has no significant effect in stimulating GH release.

The reasons for the lack of response to subsequent pulses of hGRL might be due to: (1) the depletion of the GH stores, leading to the reduced GH release following the first pulse, or (2) due to the desensitization of the ghrelin receptors in the somatotropes by 1000 nM hGRL.

Experiment II

The results of this experiment suggest that ghrelin, even at low doses such as 0.01, 0.1 and 1 nM, desensitizes its receptor(s) in goldfish gonadotropes and somatotropes and the receptors do not return to control levels even at 3 hours after the administration of the first pulse of ghrelin.

Experiment III

The results of the experiment I and II do not clearly indicate whether desensitization of the receptors is the reason for the lack of response after the administration of a second pulse of the same ghrelin peptide at 1 hour or 3 hours later. Depletion of the GH and LH stores can also lead to the lack of response found in the previous studies. In this experiment, 1000 nM of goldfish ghrelin peptides were unable to stimulate GH and LH release, while sGnRH, administered 90 minutes after the ghrelin treatment was effective in stimulating both GH and LH release from the same columns. The results of this study suggest that the lack of response after repeated administration of the ghrelin peptides is due to receptor desensitization, not due to the depletion of GH or LH stores.

References:

Orkin RD, New DI, Norman D, Chew SL, Clark AJ,Grossman AB, Korbonits M 2003 Rapid desensitization of GH secretagogue (ghrelin) receptor to hexarelin *in vitro.* J Endcocrinol Invest 26:743-747.

Camiña JP, Carreira MC, El Messari S, Llorens-Cortes C, Smith RG, Casanueva FF 2003 Desensitization And Endocytosis Mechanisms Of Ghrelin-Activated Growth Hormone Secretagogue Receptor 1a Endocrinology. October 23, 2003 doi:10.1210/en.2003-0974.

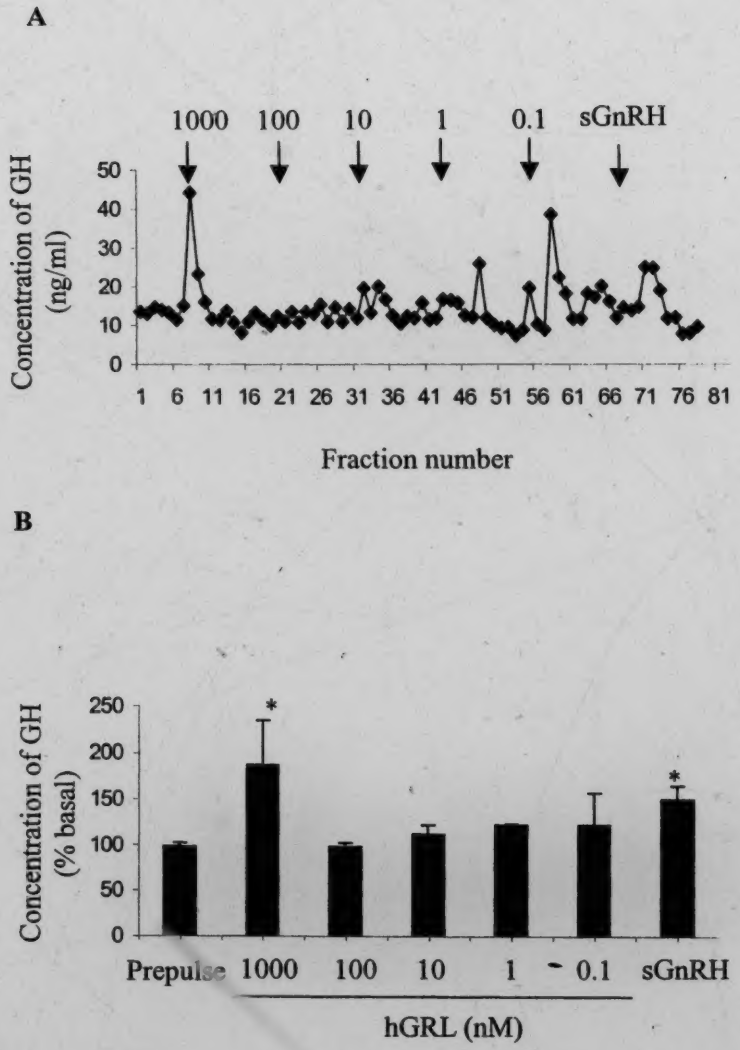


Figure A2.1. Figure A: The profile of GH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which the specific dose (nM) of human ghrelin was administered. Figure B: The pooled data from 4 different columns perifused with human ghrelin. An asterisk (*) represents values that are significantly higher than the prepulse.

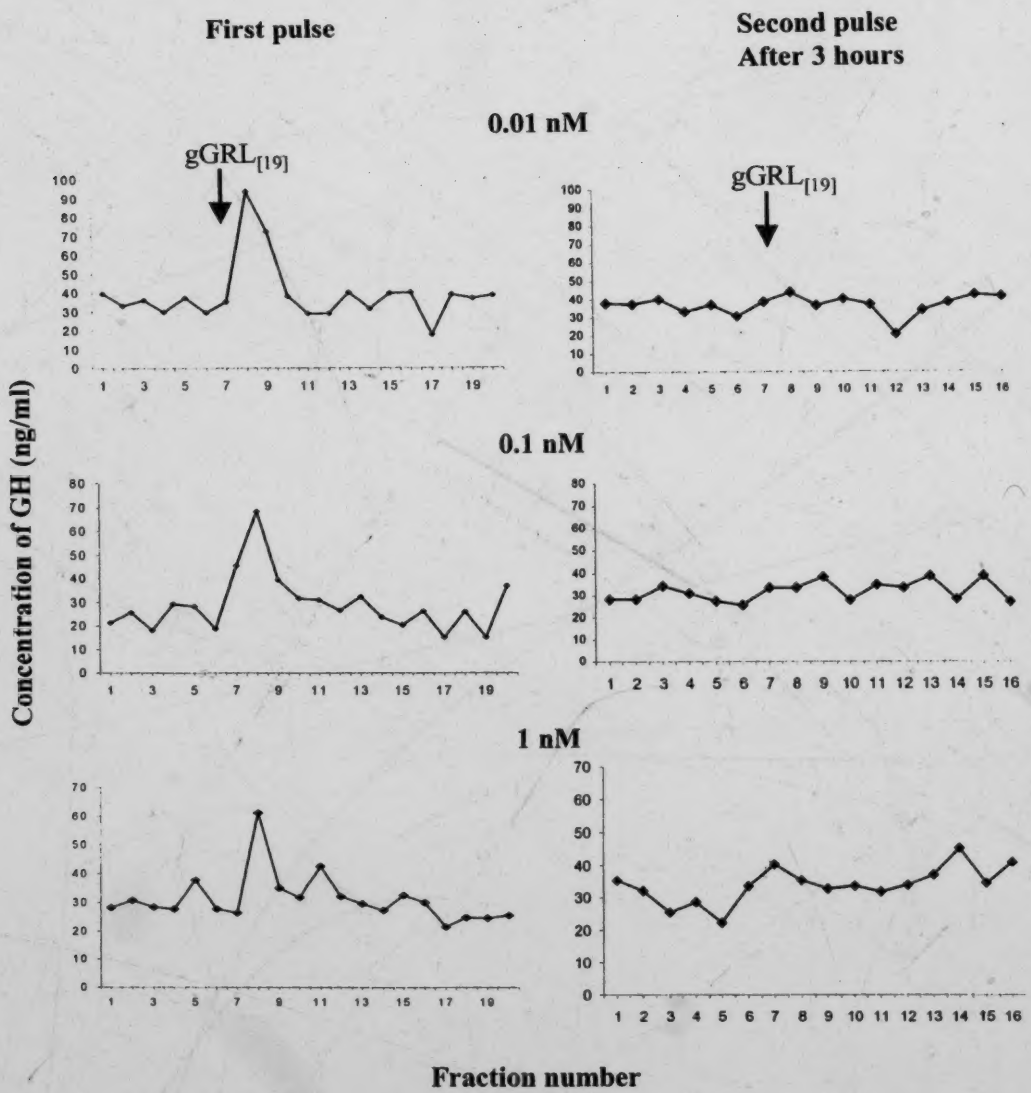


Figure A2.2. Figures in the left panel shows the profile of GH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which the specific dose (nM) of $gGRL_{[19]}$ was administered. Figures in the right panel shows the profile of GH release from the same columns 3 hours after the first pulse of $gGRL_{[19]}$.

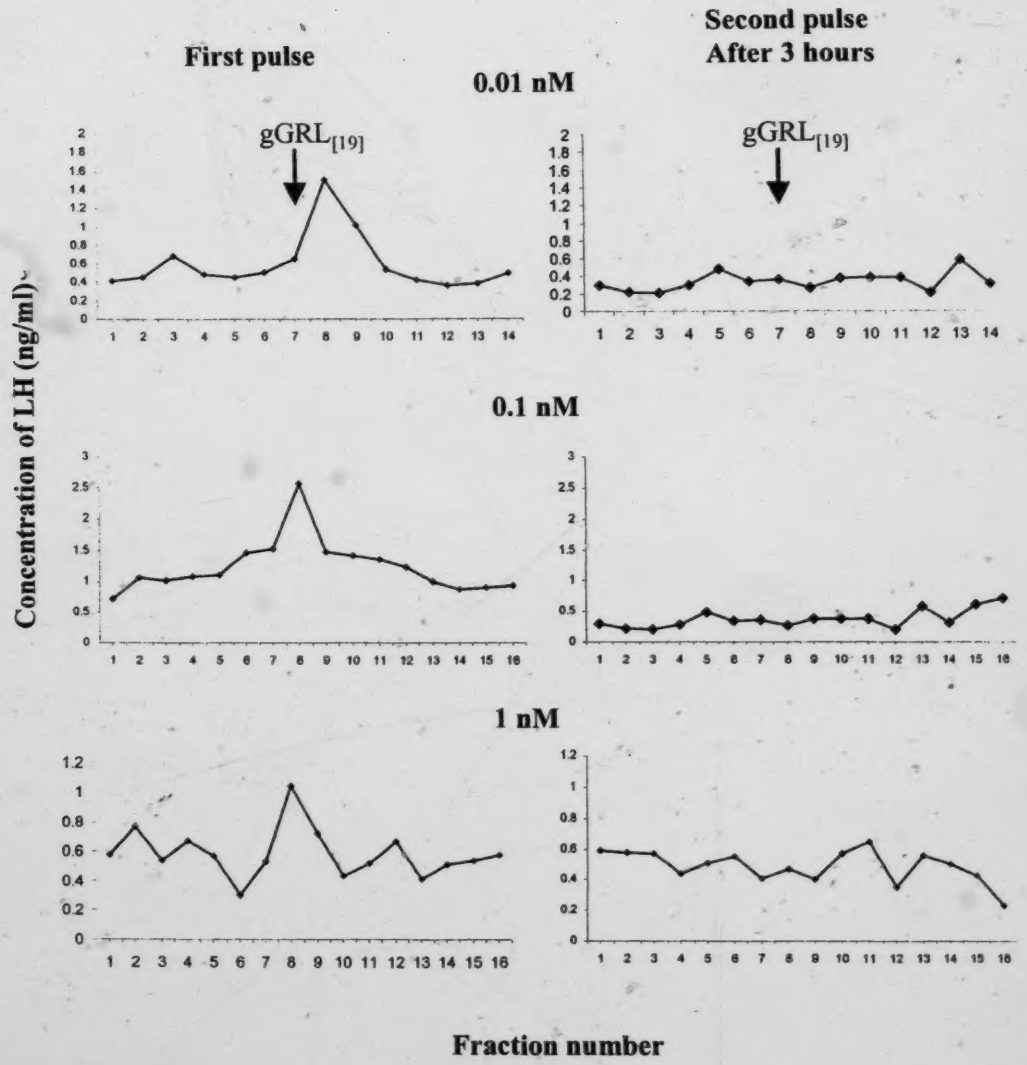


Figure A2.3. Figures in the left panel shows the profile of LH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which the specific dose (nM) of $gGRL_{[19]}$ was administered. Figures in the right panel shows the profile of LH release from the same columns 3 hours after the first pulse of $gGRL_{[19]}$.

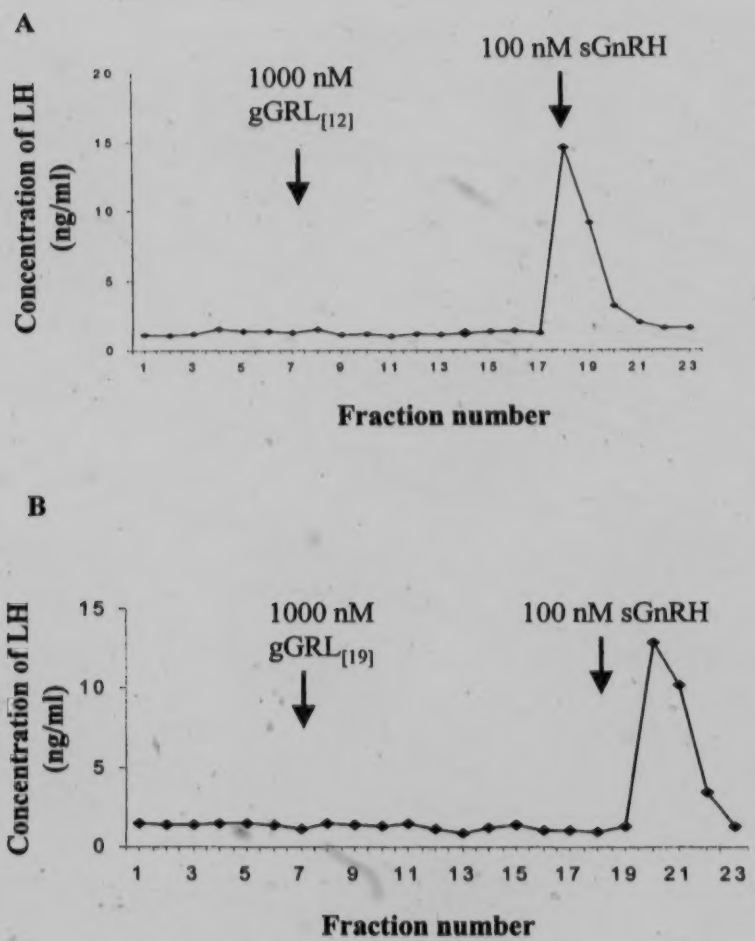


Figure A2.4. Figure A shows the profile of LH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which 1000 nM $gGRL_{[12]}$ or 100 nM sGnRH was administered. Figures B shows the profile of LH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which 1000 nM $gGRL_{[19]}$ or 100 nM sGnRH was administered.

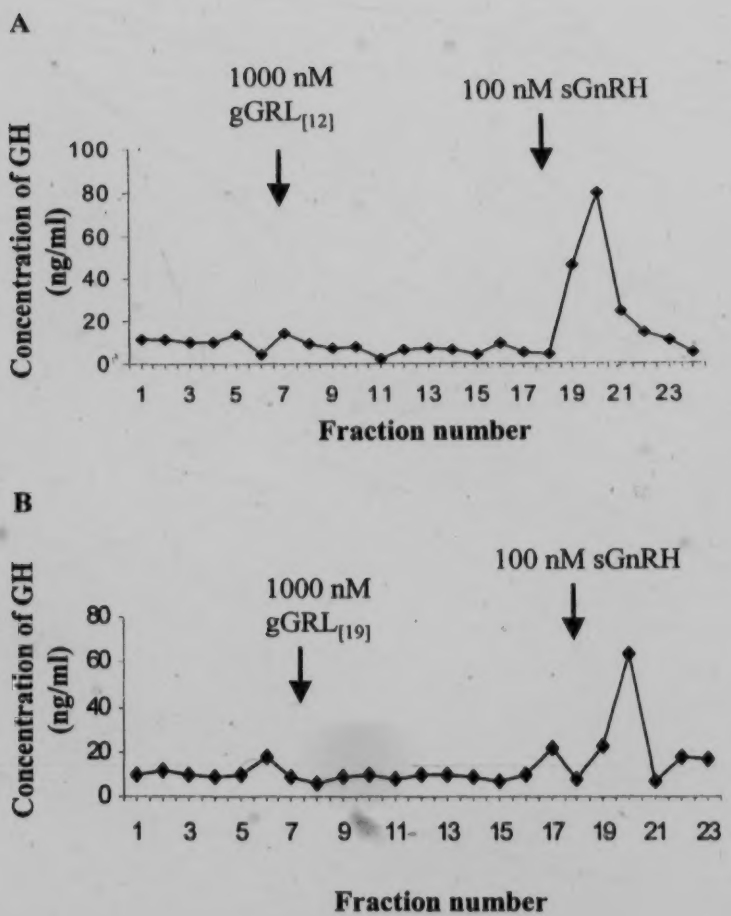


Figure A2.5. Figure A shows the profile of GH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which 1000 nM $gGRL_{[12]}$ or 100 nM sGnRH was administered. Figures B shows the profile of GH secretion from a perifusion column loaded with dispersed pituitary cells of goldfish. Arrow heads point to the fraction in which 1000 nM $gGRL_{[19]}$ or 100 nM sGnRH was administered.